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Carla Taveggia

# Distal transcriptional activators of the Myelin Basic Protein gene in Oligodendrocytes and Schwann Cells

Thesis submitted in partial fulfilment of the requirements of the  
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## ABBREVIATION USED

ACF	ATP-utilizing chromatin assembly and remodelling factor
AP-1	Activator protein 1
ARIA	Acetylcholine Receptor Inducing Activity
bHLH	Basic Helix-loop-helix
BMP	Bone Morphogenetic Protein
C/EBP	CCAAT enhancer-binding protein
CAF-1	Chromatin Assembly Factor 1
CBP	CREB-binding protein
CH	Congenital Hypomyelination
CHRAAC	Chromatin-accessibility complex
CMT	Charcot Marie Tooth
CNS	Central Nervous System
CNTF	Ciliary-Neurotrophic Factor
Dhh	Desert hedgehog
Dpp	Decapentaplegic
DRG	Dorsal Root Ganglia
DSS	Dejerine Sottas Syndrome
EGF	Epidermal Growth Factor
Egr	Early Growth Response
EMSA	Electro Mobility Shift Assay
FACT	Facilitate Chromatin Transcription
FGF	Fibroblast Growth Factor
GAP43	Growth Associated Protein 43
GFAP	Glial Fibrillary Acidic Protein
GGF	Glial Growth Factor
HDAC	Hystone deacetylase
HNPP	Hereditary Neuropathy with liability to Pressure Palsy
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein

MOG	Myelin-Oligodendrocyte Glycoprotein
NAB	NGFI-A Binding Protein
N-CAM	Neural Cell Adhesion Molecule
NGF	Nerve Growth Factor
NRG	Neuregulin
NURF	Nucleosome Remodelling Factor
OL	Oligodendrocyte
OPC	Oligodendroglial precursor cell
OSP	Oligodendrocyte-Specific Protein
P0	Protein zero
PDGF	Platelet Derived Growth Factor
PLP	Proteolipid Protein
PMP22	Peripheral Myelin Protein 22
PNS	Peripheral Nervous System
PPAR	Peroxisome Proliferator-Activated Receptor
RSC	Remodel the structure of Chromatin
SC	Schwann cell
SCE	Schwann Cell Enhancer
Shh	Sonic hedgehog
SMDF	Sensory and Motor Derived Factor
Sog	Short gastrulation
Sp-1	Specificity protein 1
SRC	Steroid Receptor co-activator
SWI/SNF	Switching/sucrose non-fermenting
TAF	TBP-associated factor
TBP	TATA Binding Protein
TGF	Transforming Growth Factor
Wnt	Wingless



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# ABSTRACT

Myelin is a multilamellar sheath that wraps the axons in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS). It is required for normal development, axonal health and to facilitate impulse conduction. MBP, one of the major myelin proteins, is synthesised by both oligodendrocytes (OL) in CNS and Schwann cells (SC) in PNS. Its synthesis is regulated primarily at the transcriptional level. MBP transcription is activated as a part of a program of coordinate myelin gene expression during development, overlapping but specific in OL as compared to SC. The nuclear proteins that bind to the MBP promoter are largely unidentified. In vitro and in vivo studies show that activation of the MBP proximal promoter requires different DNA binding sites and proteins in OL as compared to SC. Recently, transgenic mice studies identified a SC enhancer (SCE) located 9.0Kb upstream of the transcriptional start site of MBP. In this work, we compared MBP transcription mechanisms in OL and SC. Transient transfection analyses, in primary cultures of OL and SC, confirm the importance of SCE, and suggest that SCE is an activator in both cell types, but it is an enhancer only in SC. Biochemical analyses show that there are regions differentially protected by SC and OL nuclear extracts that can account for differential function. Analysis of internal deletions and transversion mutations of SCE suggests that multiple cis-acting elements cooperate to produce activation. Analyses of stably transfected SC show that chromatin upregulates SCE activation at least three fold. Co-culture of these stably transfected SC and dorsal root ganglia neurons reveal that axons markedly activate SCE, by at least two orders of magnitude. These results describe a model in which to analyse SCE biochemistry and function and identify trans-acting signals from axons that regulate MBP transcription in SC.

# Chapter 1

## INTRODUCTION

### 1.1 THE NERVOUS SYSTEM

The nervous system comprises two distinct classes of neural cells: neurons and glia and other supporting cells like those of blood vessels, meninges, immune cells and fibroblasts.

#### *NERVE CELLS*

A typical neuron contains four morphologically distinct regions: the cell body also named the perikaryon, the dendrites, the axon and the presynaptic terminal of the axon. The major characteristic of nerve cells is the ability to generate active electrical signals and each region has distinctive signalling functions. The *perikaryon* is the metabolic center of the neuron. It usually gives rise to a series of fine arborizing extensions named *dendrites* that constitute the receptive apparatus of the neurons. Some neurons develop a huge amount of dendrites, by constituting a sort of dendritic tree, while others can develop only a restricted number of them. The other characteristic neuronal structure is the *axon*, which is the conducting unit of the neuron. It is a tubular process that can extend for a long distance in the body. The high speed conduction of the action potential is facilitated by the myelin sheath which is present in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS), except at the level of the Node of Ranvier. The growth cone leads the axonal outgrowth. The growth cone moves by the elongation and contraction of microspikes, a series of pointed filopodia, which contain microfilaments, oriented parallel to the long axis of the axon. Microspikes have both a sensory function and a structural role (Davenport *et al.*, 1993). They contain microfilaments, that if destroyed by

cytochalasin B, blocks further axonal advance (Lamoureux *et al.*, 1989). In general, the axon contains little protein synthesis machinery, and thus much of the protein required in the axon and synaptic terminal must be transported down the axon after it is synthesised in the cell body. Newly synthesised proteins are assembled into organelles and transported to the presynaptic terminals by a process called axoplasmic transport. The structures that support this transport are the microtubules, which serve as guides for the transport of organelles. Two families of motor proteins are involved in trafficking molecules and organelles along the microtubules: kinesins and dyneins. They are both microtubule – activated ATPase proteins. Kinesins promote the anterograde transport, from cell body towards the extremities, while dyneins are responsible for the movement in the other direction (Hirokawa, 1998). A recent paper shows that even the growth cone of the axon is a site of protein synthesis. This protein synthesis represents local translation since it occurs even when the growth cone is physically detached from the perikaryon (Campbell and Holt, 2001).

Near its end the axon divides into many fine branches, which have specialised extremities called *presynaptic terminals*, the transmitting elements of the neuron. Through them a neuron contacts and transmits information about its own activity to the receptive surfaces of any effector cells. The point of contact is called *synapse*. The synapse is formed by the presynaptic terminal of a cell, the *receptive surface* of the other and the space between them: the *synaptic cleft*. The terminals of the presynaptic neuron sometimes contact the post synaptic neuron directly on its cell body, more commonly they make contact with dendrites. In each neuronal cell information goes from the receiving sites of the neuron (cell body and dendrites), to the region from which the impulse initiates and finally to the presynaptic site in the axon that communicates with other cells. Between nerve cells there is no cytoplasmic continuity and each cell has specific and precise connections only with some nerve cells and not with others.

On the basis of the number of processes that arise from the cell body, neurons are classified into three groups, unipolar, bipolar and multipolar. *Unipolar neurons* have one primary process that gives rise to many branches. Some of them serve as dendritic structures and others as axons and terminal structures. They predominate in the nervous system of invertebrate animals. *Bipolar neurons* have an ovoid soma that

gives rise to one process at each end: a dendrite or peripheral process and an axon or central process. The retinal bipolar cells belong to this class of neurons. *Multipolar neurons* predominate in the vertebrate nervous system. These cells have one or more dendritic processes and a single axon. In a typical multipolar cell, dendrites emerge from all parts of the neuronal body. Even within the category of the multipolar neurons the size and the shape of different cells vary greatly. Different types of multipolar cells account for all of the distinguishable neuronal types. The morphological differences among multipolar cells are due largely to variations in the number and length of dendrites and length of the axon. The number and extent of dendritic processes in a given cell correlate with the number of synaptic contacts that other neurons make on that cell, while the length of the axon reflects the signalling function of a neuron.

### GLIAL CELLS

Glial cells are found between nerve cell bodies and also between axons. The various types of glial cells are thought to serve at least six distinct functions:

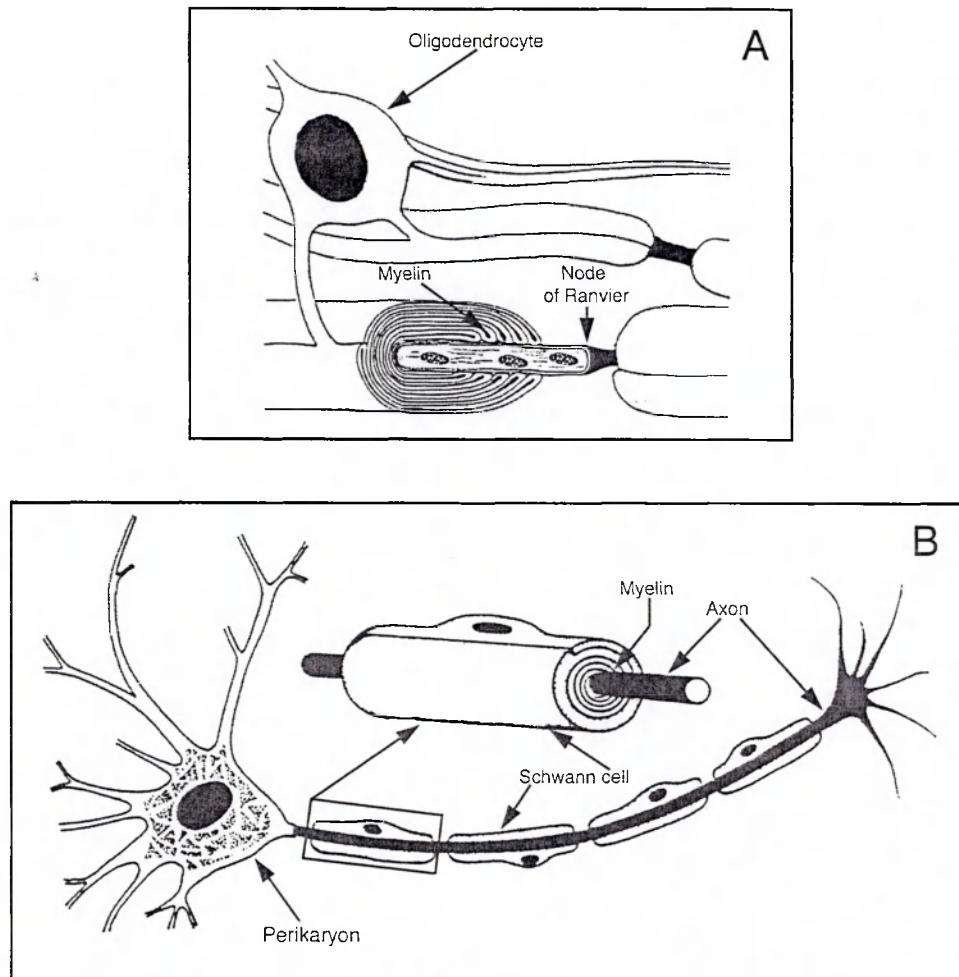
1. provide myelin;
2. provide firmness and structure to the brain. They also segregate and occasionally insulate groups of neurons from each other;
3. remove debris after neuronal death or injury;
4. buffer the  $K^+$  concentration of the extracellular space and help to remove chemical transmitters released by neurons;
5. guide the migration of neurons and direct the outgrowth of axons during development;
6. be nutritive for nerve cells.

Glial cells are generally divided into major classes: *macroglia* and *microglia*. Macroglia include myelin forming cells (Fig. 1.1): Oligodendrocytes (OL) in the CNS and Schwann cells (SC) in the PNS, astrocytes and ependymal cells which line the central canal of brain and spinal cord. Microglia include an assortment of phagocytic cells that are mobilised after injury, infections or diseases. Some studies suggest that they can play active roles during cell degeneration. They also produce trophic factors that support the development of neurons and glia (Elkabes *et al.*, 1996). They originate

from mesodermic precursors of hematopoietic lineages that enter the nervous parenchima from meninges, ventricular space or blood stream (Cuadros and Navascues, 1998). Once in the CNS they migrate to their final destination where they differentiate.

The two predominant classes of macroglial cells in the CNS are OL and astrocytes. Astrocytes are star-shaped cells with small irregular cell bodies, and many extensions that ramify between the processes of nerve cells. They are usually divided in two classes: fibrous and protoplasmic cells. Fibrous astrocytes contain many filaments and are found in the white matter areas of the CNS. The protoplasmic astrocytes have shorter processes that contain few filaments; they are located in the gray matter of the CNS and they are associated with nerve cell bodies, dendrites and particular synapses. They probably have nutritive functions; in fact their processes can contact blood capillaries on one side and neurons on the other. After injury, astrocytes and microglia remove neuronal debris and promote the sealing of the lesioned regions. They are also sensitive to small changes in  $K^+$  concentrations in the extracellular space. By taking up the extracellular excess of  $K^+$ , astrocytes can buffer  $K^+$  concentration; in this way the membrane potential of neurons is protected from depolarisation that might result if  $K^+$  accumulated after repeated neuronal firing.

The major function of OL and SC is to produce myelin respectively in the CNS and in the PNS. They form this sheath by wrapping their membranous processes around the axon in a tight spiral. One of the major differences between these two cell types is that a single OL can simultaneously envelop many axons in the CNS and make myelin around them, while SC can myelinate only a single axon whose diameter has to be larger than  $1\mu m$  (Fig. 1.1). I will discuss their developmental origins and roles, in much more detail, in section 4 where I will present the nervous system development.



**Figure 1.1**

Schematic representation showing how an oligodendrocytes (A) and a Schwann cells (B) enwrap axons. (A) In the CNS, oligodendrocytes enwrap simultaneously many axons, whereas in the PNS SC wrap only a single axon (B). The region between two myelinated tracts is the Node of Ranvier and it is specifically indicated in (A). (B) shows also a schematic representation of a nerve cell, whose cell body is the perikaryon, myelinated by SC.

## 1.2 HISTO ARCHITECTURE OF THE CNS

The anatomy of the CNS appears quite complex. The neurons of the brain are organised into layers (*cortices*) and clusters (*nuclei*), each having different functions and connections. The original neural tube is composed of a *germinal neuroepithelium*. As the cells continue to divide, the migrating cells form a second layer of cells around the original neural tube. This new layer is called the *mantle* or *intermediate zone* and the germinal epithelium is now called the *ventricular zone*. The mantle zone cells differentiate into neurons and glia. Neurons make connections among themselves and send axons away from the lumen forming another zone, with a reduced number of cells, named the *marginal zone*. The mantle zone, containing the neuronal cell bodies is often referred to as the gray matter, while the axonal, marginal layer is referred to as the white matter given the presence of myelin sheaths. This original organisation in three zones is maintained only in the spinal cord. In the brain, cell migration, selective cell death and differential neuronal proliferation produce modifications of the three zone patterns. The brain is composed of many regions; each of which can be further subdivided in several anatomically and functionally distinct areas. The major brain divisions are the brainstem, which comprises medulla, pons, and midbrain, the cerebellum, the diencephalon and the telencephalon (Fig. 1.2).

Information coming from neurons is always modified at every step, and the output of one stage of a functional system is rarely the same as its input. Information may be attenuated or amplified at any stage. At each stage a single neuron typically receives inputs from thousands of presynaptic neurons, and all these influences govern the output of the neuron to the next stage. Although a variety of neurons are involved at each stage in information processing, neurons can generally be divided in three classes: *sensory neurons*, *motor neurons* and *interneurons*. Sensory neurons convey information from the periphery of the cell body to the nervous system; instead motor neurons carry commands from the CNS to muscles and glands. The interneurons convey information to the next stage in the system and they can be ulteriorly subdivided into projection and local interneurons. Axons leaving one component of a functional system are bundled together in a *pathway* that projects to the next component.



### THE NEUROEPITHELIUM

The *germinal neuroepithelium* is one cell layer thick and it is composed of rapidly dividing neural stem cells. All the cells of the neuroepithelium extend from the luminal surface of the neural tube to the outside surface, but the nuclei of these cells are at different levels. Radiolabelling by incorporation of nucleotide analogs showed that these cells continue to divide while they remain in this layer of cells. Shortly thereafter certain cells stop dividing and they start to migrate and differentiate outside the neural tube (Jacobson, 1968). When an intraepithelial stem cell divides, the daughter cell adjacent to the lumen remains connected to the ventricular surface, while the other cell migrates away (Chenn and McConnell, 1995). The time of this vertical division is the last time at which a neural stem cell divides and is called the “neuron’s birthday”. Different types of neurons and glial cells undergo this last division at different times. Labelling at different times during development showed that cells with the earliest last division are those that migrate shorter distances. On the contrary the cells with later birthday are those that migrate through these layers to form the more superficial layers of the cortex. Subsequent differentiation depends on the positions that these neurons occupy once outside the germinal neuroepithelium (Jacobson, 1991).

### THE SPINAL CORD

It is the most caudal part of the CNS (Fig. 1.2). The spinal cord receives sensory information from skin, joints and muscles of trunk and limbs and contains the motor neurons responsible for both voluntary and reflex movements. It is divided into gray matter and surrounding white matter. The gray matter contains nerve cell bodies, and it is typically divided into ventral and dorsal horns. The dorsal horn has sensory neurons that receive information from the periphery, while the ventral horn contains motor neurons. The white matter is made by longitudinal tracts of myelinated axons. The nerve fibers that link the spinal cord to muscles and sensory receptors in the skin are bundled in 31 pairs of spinal nerves in human, each of which have a sensory component that emerges from the dorsal root and a motor component that emerges from the ventral root.

### *THE BRAINSTEM*

It can be subdivided in medulla, pons and midbrain (Fig. 1.2). It is continuous with the spinal cord and contains distinct nerve cell clusters that contribute to a variety of sensory and motor systems. The sensory input and motor output are performed by 12 cranial nerves that are functionally similar to spinal nerves. The brainstem is involved in sensation and motor control of the head, neck and face. Neurons in the brainstem control many parasympathetic reflexes such as blood pressure. It contains ascending and descending pathways that carry sensory and motor information to other divisions of the CNS.

### *THE CEREBELLUM*

It receives sensory input from the spinal cord and motor information from the cerebral cortex (Fig. 1.2). It is important for maintaining posture and for co-ordinating head and eye movements and it is also involved in the movements of muscle and in learning motor ability. In it there are also some functions related with language and other cognitive aspects.

### *THE DIENCEPHALON*

It is composed of the thalamus and the hypothalamus (Fig. 1.2). The thalamus is important for transfer of sensory information from the periphery to processing regions of the cerebral hemispheres. It is particularly important because it determines which sensory information reaches consciousness in the neocortex. The hypothalamus lies ventral to the thalamus and regulates several behaviours that are essential for homeostasis and reproduction. It is an essential component of the brain systems mediating emotions.

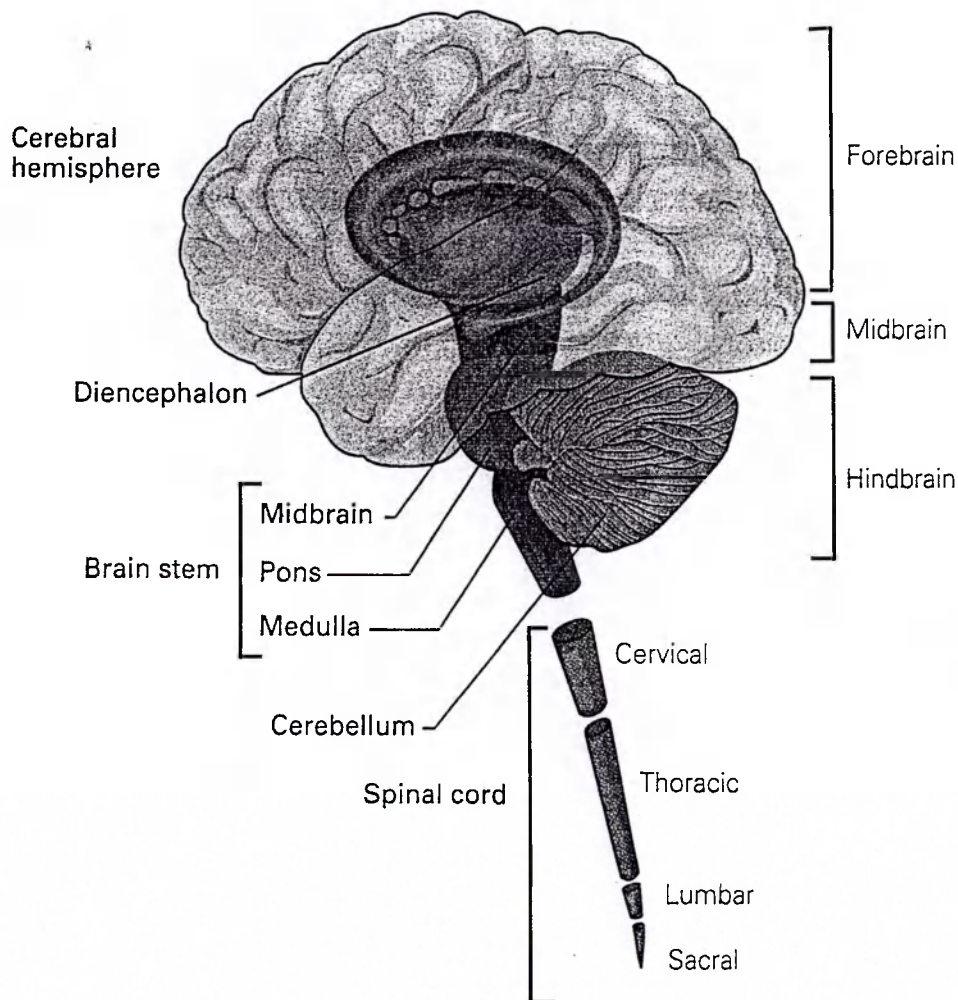
### *THE TELENCEPHALON*

It constitutes the largest region of the human brain. It is formed by the cerebral cortex, the underlying white matter, the basal ganglia, the amygdala and the hippocampus (Fig. 1.2). The telencephalon, also identified as cerebral hemispheres, is involved in all cognitive functions, including memory and emotion. The two hemispheres are interconnected by the corpus callosum, a set of fibers that connect

symmetrical regions in both hemispheres. The major characteristic of the human brain is given by the cerebral cortex. The number of neurons in it is one of the crucial determinants of cortex's capacity for information processing. It is organised in six functional layers numbered from the outer surface of the cortex (*pia mater*) to the white matter. Each layer differs from the other in its functional properties, the type of neurons and the sets of connections they make. The neocortex receives inputs from the thalamus and from other cortical regions on both sides of the brain. The output of the neocortex is also directed to several brain regions, including other regions of the neocortex, the thalamus and the spinal cord. Two classical types of neurons principally form the neocortex: projection neurons and interneurons. Projection neurons typically have pyramidal shaped cell bodies. They are mainly located in layer III, V and VI and use the excitatory acid glutamate as a primary neurotransmitter. Interneurons use the inhibitory GABA neurotransmitter, they constitute approximately 20-25% of the neurons in the neocortex and they are located in all layers.

Neurons in the neocortex are not only distributed horizontally in layers, but they are also positioned in columns that trasverse the layers. Neurons within a particular column tend to have very similar response properties, presumably because they form a local processing network. Neither the vertical nor the horizontal organisation of the brain is clonally specified. The developing cortex, in fact, originates from a mixing of cells that derive from different neural stem cells. After their final mitosis, most of the neuronal precursors generated in the ventricular zone migrate along glial processes to form the cortical plate. The fate of neuronal precursors from older brains are more restricted. While the neuronal precursor cells formed early in development can become any neuron, later precursor cells give rise to neurons only in outer layers (Frantz and McConnell, 1996). Young neurons reach their final destination, in the developing brain, through the interactions with radial glia. This mechanism, called *glial guidance*, is determined by a series of events, involving reciprocal recognition between glia and neuroblasts (Komuro and Rakic, 1992, Hatten, 1990). Radial migration is not the only mechanism that determines axonal movements; in fact axons can migrate also laterally from one functional region of the brain to another (O'Rourke *et al.*, 1992). The specification of the cortical areas into functional domains occurs after neurogenesis. Once the cell arrives at its final destination it becomes organised in specific brain nuclei

(Matsunami and Takeichi, 1995). Not all cells become glial cells or neurons and migrate; some cells remain as integral components of the neural tube giving rise to the ependymal cells, which are the precursors of neurons and glial cells.



**Figure 1.2**

Schematic representation of the Central Nervous System. The CNS can be divided into many regions. Each region can be further sub-divided into other anatomical and functional parts. In particular the spinal cord is sub-divided into sacral, lumbar, thoracic and cervical districts, from which originate the spinal nerves. The brainstem is composed of medulla, pons and midbrain (Modified from Kandel E.R.; Schwartz J.H. and Jessell T.M. Principles of Neural Science; 4<sup>th</sup> edition; published from McGraw-Hill, New York, 2000)

### CENTRAL NERVOUS SYSTEM STEM CELLS

Neural stem cells are defined as multipotent, self-renewing progenitor cells, and they have been identified both in the CNS and in the PNS. In the CNS, neural stem cells should differentiate into neurons, astrocytes and OL. Early studies led to the isolation of stem-like cells from many regions of the embryonic mammalian CNS (Kilpatrick and Bartlett, 1993). Other analyses showed that neural stem cells can be isolated also from adult CNS (Reynolds and Weiss, 1992). In particular adult neural stem cells have been found in the hippocampus and in the subventricular zone (Johansson *et al.*, 1999) including spinal cord (McKay, 1997). Neural stem cells can be isolated also from more primitive cells, including embryonic stem cells. It was in fact shown that mouse embryonic stem cells can be induced to differentiate into a population of cells enriched in oligodendrocyte precursors (Gage, 2000; Brustle *et al.*, 1999).

These studies showed that stem cells associated with the lateral ventricles of the brain give rise to immature neurons that migrate to the olfactory bulb, where they differentiate and integrate as interneurons. In adherent cultures, CNS stem cells produce large clones containing neurons, glia and also other stem cells. In vitro these cells can be expanded in defined media containing both EGF and FGF-2 (Gritti *et al.*, 1996). Analysis of these clones suggested that stem cells are prevalent at early stages of embryonic development. Other studies suggest that stem cells isolated from different neural regions generate only particular sub-types of neurons. The pattern emerging is that vertebrate neural stem cells are regionally specified. Moreover it seems that neural stem cells acquire, during development, temporal information. In fact some early neural tube stem cells produce both CNS and PNS stem cells, suggesting that there is a common progenitor for two separate stem cell lineages (Mujtaba *et al.*, 1998). On the contrary stem cells purified in later stages of development confirm that not only there are different stem cell lineages between CNS and PNS, but also the neurogenic capacity in each lineage declines with age (Lois and Alvarez-Buylla, 1994).

### 1.3 HISTO ARCHITECTURE OF THE PERIPHERAL NERVE

Individual nerve fibers are protected from the environment by a compact connective tissue matrix, consisting of three layers: the epineurium, the perineurium and the endoneurium (Fig. 1.3).

#### THE EPINEURIUM

The epineurium is mainly composed of connective tissue that surrounds the fascicles of the peripheral nerve. It is the only part of the PNS that contains a lymphatic capillary network connected to regional lymphnodes. The cellular components of the epineurium are fibroblasts lacking a basal lamina, while the non-cellular part consists mainly of collagen type I and III. In the epineurium there are also variable amounts of fat whose function is to protect the fascicles against damage by compression. Blood is supplied via the *vasa nervorum*, a network of arterioles and venules, which have branches through the perineurium and the endoneurium.

#### THE PERINEURIUM

The perineurium is a multilayered cellular sheath, usually in the number of six to eight, surrounding individual fascicles of the nerve. The epithelial cells that form these sheaths originate from the mesenchyme surrounding nerve fibers (Bunge *et al.*, 1989). The layers consists of concentric flattened epithelial cells with a basal lamina connected to each other by tight junctions and gap junctions. Both the inner and the outer cell layer of the sheath produce a basal lamina consisting of a fine network of collagen type IV fibers, fibronectin, and heparin sulfate proteoglycan. In the perineurium there is a selective transport mechanism that restricts transfer of molecules into the endoneurium. The presence of pinocytic vesicles and caveolae in perineurial cells together with high ATPase and creatine kinase activity confirm this active transport mechanism. The other function of the perineurium is to protect the nerve from damage through a cushioning function obtained by collagen layers. SC can directly influence the formation of the perineurium through the action of the signalling molecule *Desert hedgehog* (*Dhh*) (Parmantier *et al.*, 1999). This molecule is expressed by SC precursors during development. *Dhh* null mice revealed that all

components of the connective tissue sheath were defective, whereas myelination occurred normally. Both the perineurium and the epineurium were disorganised and uncompacted; the perineurial cells were ectopically located in the endoneurial space and failed to express the gap junction protein Connexin 43. Ultrastructural analysis showed that tight junctions were abnormal despite the fact that proteins were normally located. In addition the endoneurium was accessible to bacteria showing the complete lack of the barrier function of the perineurium. This and other studies strongly suggest that SC are important regulators of PNS development. In fact, beside myelination, they can control the formation of the connective tissue surrounding the nerve, and the distribution of ion channels along nerve fibers (Riethmacher *et al.*, 1997) and axon survival (Vabnick *et al.*, 1996; Salzer, 1997; Jessen and Mirsky, 1999).

### *THE ENDONEURIUM*

It mainly consists of a thick layer of collagen fibrils running parallel along the nerves and it constitutes the intrafascicular compartment of the nerve. Its function is presumably mainly supportive and protective. This collagen layer takes up almost 50% of the intrafascicular space, and increases as the animals age. Embedded in the endoneurium are axons ensheathed by SC. SC, together with endoneurial fibroblasts, are the major cellular component of the intrafascicular space. Endoneurial fibroblasts are the main producers of the collagen that is present in this structure.

### *NON MYELINATED PERIPHERAL AXONS*

Most non-autonomic peripheral nerves in vertebrates consist of a mix of myelinated and non myelinated fibers. In contrast, sympathetic and parasympathetic nerves contain predominantly non myelinated fibers. The decision to become a myelinating versus non-myelinating SC is determined by the axonal diameter; in fact, under normal conditions, only axons with a diameter bigger than 1µm will become myelinated, whereas smaller axons will not (see chapter 5: SC-axon reciprocal influences). Non myelinating SC segregate a variable number of small caliber axons, from a single axon up to twenty, by sending out cytoplasmic processes in between. The way in which a SC can enwrap the axons in a bundle of unmyelinated fibers can vary depending on nerves and between species. Usually many axons are packed in a

common compartment limited by SC tongues. Some axonal isolation is achieved when SC tongues divide the bundles and separate individual axons. Although several axons continue to stay in the same bundle, they can not contact other axonal surfaces. More frequently SC processes cover the whole surface of unmyelinated axons. When a SC process fails to cover part of the surface of a peripheral axon, there is still a layer of basal lamina separating the axon from the endoneurium.

#### *PERIPHERAL NERVOUS SYSTEM STEM CELLS*

Self renewing progenitors of neurons and glia were first identified by in vitro subcloning experiments in the PNS (Stemple and Anderson, 1992). They originate from the lateral edges of the neural plate. Multipotent progenitors are derived from post migratory neural crest populations, but they are more restricted in the types of derivative they can generate. Various multipotent neural crest-derived populations have been isolated also from rat sciatic nerve during development (Morrison *et al.*, 1999). As for the CNS neural stem cells, also the PNS neural stem cells are temporally specified.

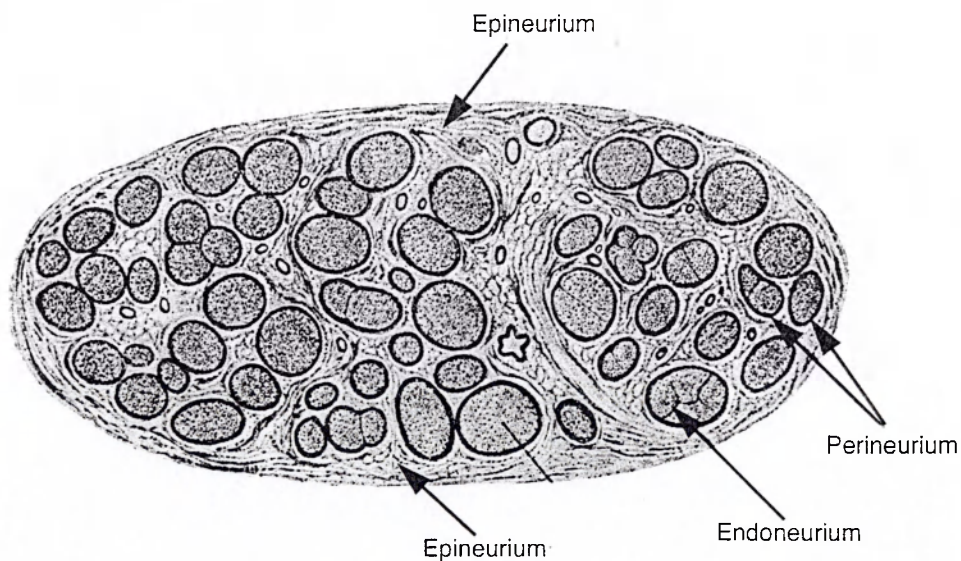
By FACS sorting, different populations of multipotent self-renewing stem cells were isolated based on the presence or absence of the peripheral Myelin Protein Zero (P0) and the Nerve Growth Factor Receptor p75. Progenitors expressing p75 in the presence or absence of P0 behave in an identical manner as the originally identified neural crest stem cells, when grown in the presence of Glial Growth Factor-2 (GGF-2), Bone Morphogenetic Proteins-2/4 (BMP-2/4) or Transforming Growth Factor  $\beta$ 2 (TGF $\beta$ 2). However these clones, when grown in standard culture conditions are fate restricted.

Two different studies purified and studied in cell culture clones of neural crest cells expressing P0. In the first case Lee and co-workers, found that P0 expressing cells represent a subpopulation of cells predetermined to form glia (Lee *et al.*, 1997). In the other study, performed by Hagedorn and collaborators, they purified a multipotent precursor population expressing p75, P0 and Peripheral Myelin Protein 22 (PMP22) from Dorsal Root Ganglia (DRG). When these cells are treated with TGF $\beta$ 2 they generate muscle like cells, while communities of P0/PMP22 progenitors generate neurons that undergo apoptosis (Hagedorn *et al.*, 1999). The fact that P0 expressing



cells can give rise also to neurons and myoblasts, either in the presence or absence of instructive signals, suggesting that early P0 expression is not restrictive for neural crest to generate glial cells, at least in vitro (Morrison *et al.*, 1999). Whether this is actually the case in vivo still has to be determined.

Before entering into the mechanisms that control the development of both CNS and PNS, I will introduce a general section on transcriptional mechanisms. In fact formation and specification of both CNS and PNS are strictly regulated by many transcription factors.



**Figure 1.3**

Transverse section through a sciatic nerve. In the figure the main anatomical structures of a peripheral nerve are indicated: the epineurium, the perineurium and the endoneurium (From Balboni *et al.* *Anatomia Umana*, 3°vol.; Edizioni Ermes Milano, 1991.)

## 1.4 TRANSCRIPTION FACTORS

Biochemical and genetic analysis of many model organisms has identified a huge number of proteins responsible for transcriptional control; nevertheless the complexity of the general machinery has not been completely dissected. Despite these advances, little is known on the detailed mechanisms by which individual genes are turned on or off. This limitation is principally due to the fact that many of these studies were aimed at the identification of transcription factors in a context different from the *in vivo* situation. In fact a general approach taken to study transcriptional regulation has been to first identify a protein binding site by placing single or multiple copies of the site upstream of heterologous promoter. This approach has the advantage of a simple and sensitive analysis. However this kind of study provides information concerning what a transcription factor has the potential to do but does not necessarily reveal the specific contribution of a factor in the natural context. In fact a transcription factor can be influenced either by its position relative to other factors or by the abundance of transcriptional co-factors in the cell. Recent evidence suggests that there is an ordered progression of events leading to RNA synthesis *in vivo* and that an highly structured eukaryotic nucleus may be important in orchestrating transcription.

The first indication of the complexity of eukaryotic transcription was suggested by the finding of three distinct and separate enzymes for RNA synthesis (Roeder and Rutter, 1969):

- the RNA polymerase I which transcribes the ribosomal RNA (rRNA)
- the RNA polymerase II which transcribes the messenger RNA (mRNA)
- the RNA polymerase III which transcribes the transfer RNA (tRNA) and the small 5S rRNA.

Usually the analysis of transcriptional regulation focuses on the identification of components fundamental for correct gene regulation. This can be addressed first at the transcriptional start site, where the RNA polymerase II holoenzyme is bound. Nevertheless it is important to study also *cis*-acting elements (i.e. DNA sequences) and *trans*-acting factors (i.e. transcription factors) that determine the regulation of gene expression during development or even during adult life.

The characterisation of the DNA sequences involved in gene regulation has led to the mapping of promoter and enhancer sequences. Core promoter elements that direct transcription by RNA polymerase II include the TATA box, the initiator and the downstream promoter element (Matsui *et al.*, 1980; Smale and Baltimore, 1989). Many genes have enhancer elements in the proximal promoter, but many gene-specific signals are located far upstream of the transcription start site, in distal enhancer sequences (Carlsson *et al.* 1993). Eukaryotic genes, in fact, contain complex arrays of specific DNA sequences that combine common core promoter elements, with many gene-specific enhancer elements and cooperate to define specific expression patterns (Dyana, 1989). After these discoveries it was hypothesised that enhancer-bound activators would directly recruit RNA polymerase II to promoters in nucleosome-free regions of the template to initiate RNA synthesis (Ptashne and Gann, 1997).

The purification of transcription factors from mammalian cells with biochemical techniques revealed the existence of large families of sequence specific DNA binding proteins. They include the Specificity Protein-1 (Sp-1), the Activator Protein-1 (AP-1), the CCAAT Enhancer-Binding Protein (C/EBP), steroid receptors and tissue specific transcription factors (Dyana and Tjian, 1983; Mitchell and Tjian, 1989). In the mean time a series of proteins necessary to program a functional basal machinery were characterised. They comprise the TATA – binding protein (TBP), and general transcription factors such as TFIIA, TFIIB, TFIID, TFIIE, TFIIIF and TFIIH (Conaway and Conaway, 1993). The availability of in vitro transcription reactions as well as biochemical techniques, helped in establishing a general order of assembly of these factors for the formation of an active pre-initiation complex in vitro (Buratowski *et al.*, 1989).

Using the same techniques it was possible to characterise TFIID, which at the beginning was thought to be equivalent to TBP (Hahn *et al.*, 1989). However, purified TFIID contains not only TBP but also some ancillary factors, named TBP-associated factors (TAFs), which are critical to mediate the responsiveness (Pugh and Tjian, 1990; Dynlach *et al.*, 1991; Tanese *et al.*, 1991). Based on these experiments it was postulated that a new class of molecules, called co-activators or adaptors are necessary to convey the information instructed by the trans-acting factors to the polymerase II machinery. Many biochemical and genetic analyses characterised most of the

components of the basal machinery as well as co-activators and co-repressors which modulate different transcription factor functions (Tyler and Kadonaga, 1999; Glass and Rosenfeld, 2000). In addition many studies showed that not only sequence-specific DNA binding regulators have cell type specific expression, but also co-regulators may have tissue-restricted expression. For example the human TAF105 is highly expressed in B cells (Dikstein *et al.*, 1996). Co-activators can also play a role in regulating the cross-talk between tissue-specific transcription factors which can lie also far upstream of the start site of transcription and the RNA polymerase II machinery. Together with differential expression patterns, additional controls came from the modification of regulators, or co-regulators, via cellular signal transduction pathways, allowing transcription factors to target different subsets of basal co-regulators at different genes or in different cell types. Consistent with the fact that many genes are regulated by mixing different types of activators and repressors, many transcriptional co-factors are constituents of multi-subunit complexes, such as TFIID.

Transcriptional co-activators can be subdivided in three different classes:

- Co-factors that are intrinsic or intimately associated with components of the core machinery. They are also involved in promoter recognition and enzymatic functions. In this category belong the TAFs and TFIIA.
- Activator and repressor adaptors preferentially associated with activator or repressor molecules. They modulate DNA binding and target other co-regulators or other core machinery. Examples of this category are Groucho, Notch and also viral co-regulators E1A and VP16.
- A family of large multi-subunit co-activators structurally related but divergent in function. Some of them can interact with RNA polymerase II; others appear to have inherent enzymatic functions or chromatin selective properties. In this category belong: the yeast Mediator, and the human Srb mediator.

Co-regulators also include components of the chromatin remodelling machinery and the histone acetylases and deacetylases. One of the major problems that a transcription factor has to overcome is DNA accessibility. Eukaryotic cells contain a huge amount of genetic information and to control such a large genetic load, eukaryotes have organised colinear DNA into chromosomes each packaged into chromatin, the minimal unit of which has been defined as the *nucleosome*. The nucleosome consists of

146bp of DNA wrapped in superhelical turns around a histone octamer containing two molecules of histones H2A, H2B, H3 and H4. Adjacent to the core nucleosome, there is another histone, histone H1, which is located at the linker DNA between two nucleosomes and whose function is probably to seal DNA in the nucleosome by binding at the point where the nucleic acid enters and leaves. This unit is repeated once every  $200\text{bp} \pm 40\text{bp}$  as a nucleosomal array in chromosomal DNA. The functional consequence of this packaging is to restrict the access of DNA to a variety of DNA binding proteins that regulate gene activity. Variable degrees of DNA sequence accessibility exist within chromatin throughout the cell cycle to permit and regulate biological processes such as DNA replication, gene expression and cell division. Previous biochemical and genetic evidence suggested that nucleosomes are repressive for DNA transcription (Pennisi, 1997). Instead it is becoming clear that chromatin is a dynamic and active structure in regulating transcription of the eukaryotic genome. In fact several distinct mechanisms are involved in modulating the chromatin; some of them function as motors to disrupt nucleosomes or as enzymatic machinery to chemically modify histones.

### *CHROMATIN REMODELLING*

The mechanisms by which specific genes are activated in chromatin have been extensively investigated in a variety of biochemical and genetic systems. From these studies it has emerged that chromatin remodelling and transcriptional activation are separate processes that can be regulated by distinct proteins or subunits (Pazin, 1994). There are five chromatin remodelling complexes, identified to date: Switching/Sucrose non-fermenting (SWI/SNF), Remodel the Structure of Chromatin (RSC), Nucleosome Remodelling Factor (NURF), Chromatin-Accessibility Complex (CHRAC) and ATP-utilising Chromatin assembly and remodelling factor (ACF). They are multi-subunit complexes whose molecular weight ranges from 2MDa to 0.5MDa. They can disrupt nucleosomes through ATP hydrolysis. Their action results in a facilitation of factors binding, in the case of SWI/SNF, NURF, and ACF or in transcription of chromatin-assembled genes for NURF and ACF. These complexes are distributed in all eukaryotes from yeast to mammals and they are both functionally and mechanistically distinct.

SWI/SNF is a multi-subunit complex formed by 9-12 subunits, present in yeast and mammals. It facilitates the formation of a ternary complex composed of DNA, histones and activators, which results in a destabilisation but not the loss of nucleosomes. SWI/SNF is required only transiently to generate a stable chromatin structural change in the presence of transcription factors (Owen-Hughes *et al.*, 1996).

RSC is an abundant complex in yeast and it is encoded by essential genes. It is composed of at least of 15 subunits. RSC shows similarities with SWI/SNF in terms of subunit composition, but it is 10-fold more abundant and essential for mitotic growth (Cairns *et al.*, 1996).

NURF is a chromatin-remodelling complex present in *Drosophila* and is composed of 4 subunits (Tsukiyama and Wu, 1995). It is required for chromatin remodelling for GAGA factors and it facilitates transcription from pre-formed chromatin templates. After chromatin remodelling NURF is no longer required to maintain an active promoter configuration. It functions with a variety of proteins containing distinct DNA binding domains to remodel nucleosomes (Mizuguchi *et al.*, 1998). Recently it was shown that NURF can alter nucleosomal structure by interacting with the histone H4 tail (Georgel *et al.*, 1997).

CHRAC is a chromatin remodelling complex purified in *Drosophila*, it is composed of 5 subunits and in addition to ATPases it also contains topoisomerase II (Varga-Weisz *et al.*, 1997). It reconfigures chromatin by increasing its general accessibility and converting irregular nucleosomal arrays to physiologically spaced structures. This is the first complex shown to contain activities that modulate both nucleosome structure and DNA topology. The role of topoisomerase II in CHRAC is not completely clear; it is possible that it functions to direct CHRAC to a particular chromosomal site.

ACF is composed of 4 subunits with a molecular weight of 220KDa, it was purified in *Drosophila* and it is required for nucleosome assembly and periodicity. ACF assembles nucleosomes, in combination with an histone chaperone, Chromatin Assembly Factor 1 (CAF-1), and remodels pre-formed chromatin to facilitate protein binding and DNA transcription. Both the assembly and remodelling functions are ATP dependent (Ito *et al.*, 1997). This is the first remodelling complex that was shown to directly activate transcription of chromatin template.

The other remodelling complex identified to date is Facilitate Chromatin Transcription (FACT). This a 230KDa complex composed of 2 subunits and purified from humans. This complex is quite distinct from the others because it does not facilitate transcriptional initiation or require ATP hydrolysis (Orphanides *et al.*, 1998).

All these studies suggest that chromatin remodelling of the promoter region is one of the critical steps in transcription but it is not sufficient for gene activation unless coupled with activities that favour efficient elongation through nucleosomes.

### ACETYLATION AND DEACETYLATION

The other mechanism involved in transcriptional regulation is acetylation and deacetylation. These modifications occur at specific lysine residues at the amino terminus of histone tails. Usually hyperacetylation of these residues favours active transcription of DNA, while deacetylation is considered to be an inhibitory mechanism (Kwok *et al.*, 1994). There are many histone acetylases such as CREB-binding protein (CBP)/p300, the yeast transcription factor GCN5, P/CAF, and the Steroid Receptor Co-activator-1 (SRC-1) (Ogryzko *et al.*, 1996; Yang X.Y. *et al.*, 1996; Spencer *et al.*, 1997), as well as histone deacetylases HDAC-1, HDAC-2 and sir2 (Kadosh and Struhl, 1997; Zhang Y. *et al.*, 1997; Imai *et al.*, 2000). In yeast there is now evidence that many acetyltransferases act in concert with large complexes to provide a physical connection between acetylation and transcription (Grant *et al.*, 1997).

The fact that histone deacetylation is connected with repression of transcription became relevant with the discovery that the human HDAC-1 is an homologue of the yeast transcriptional regulator Rpd3 (Taunton *et al.*, 1996). Ablation of this gene, in fact, results in an increased level of histone acetylation and hyper activation of the CUP1 gene. In yeast, even histone deacetylases can be part of complexes. Several papers have linked specific transcriptional repressors, such as YY1, with histone deacetylation (Yang W-M. *et al.*, 1996). However with the exception of YY1, transcriptional repressors do not directly interact with deacetylases. Instead co-repressors such as sin3 and SMRT usually mediate the association.

All these studies suggest that the precise gene regulation is facilitated by the specific recruitment of appropriate complexes, containing either acetyltransferases or deacetylases. It is possible to formulate many regulatory mechanisms:

- histone acetylation/deacetylation could modulate the structure of individual nucleosomes making DNA sequences accessible to the basal machinery of RNA polIII transcription;
- acetylation of histones or other proteins could act as a specific signal, similar to phosphorylation, and promote recognition by other factors;
- acetylation and deacetylation of histones could affect higher order chromatin compaction.

### HOW IS THE TRANSCRIPTIONAL MACHINERY ORGANISED?

In order to explain how the assembly of the transcription machinery is achieved, in particular regarding the core machinery, two models were proposed: a stepwise model and holoenzyme recruitment.

The stepwise model proposes an ordered assembly of transcription factors on the basis of the formation of active transcription complexes in vitro. It was in fact observed that to get efficient promoter binding, as well as the transcription initiation from naked DNA, the stepwise addition of purified basal factors is required (Buratowski *et al.*, 1989). The steps that lead to the formation of this complex are:

- the formation of a complex containing TFIID, TFIIA and TFIIB able to recognise and to bind to the TATA box;
- a more stable complex containing in addition also hypophosphorylated RNA polymerase II and TFIIF;
- an activated open complex formed by the addition of TFIIIE and TFIIH;
- the clearance of the promoter and nascent RNA synthesis upon hyperphosphorylation of the RNA polymerase II carboxy terminal domain.

The holoenzyme recruitment model was proposed when certain preparations of RNA polymerase II were co-purified with subsets of the basal machinery including some co-regulators, chromatin remodelling factors and proteins involved in DNA replication and repair (Koleske and Young, 1994; Maldonado *et al.*, 1996). One possible advantage of the holocomplex is to bypass the limited cellular concentration of individual transcription factors. On the other hand the recruitment of a pre-formed complex does not fit well with the observation that animal cells need a vast diversity.

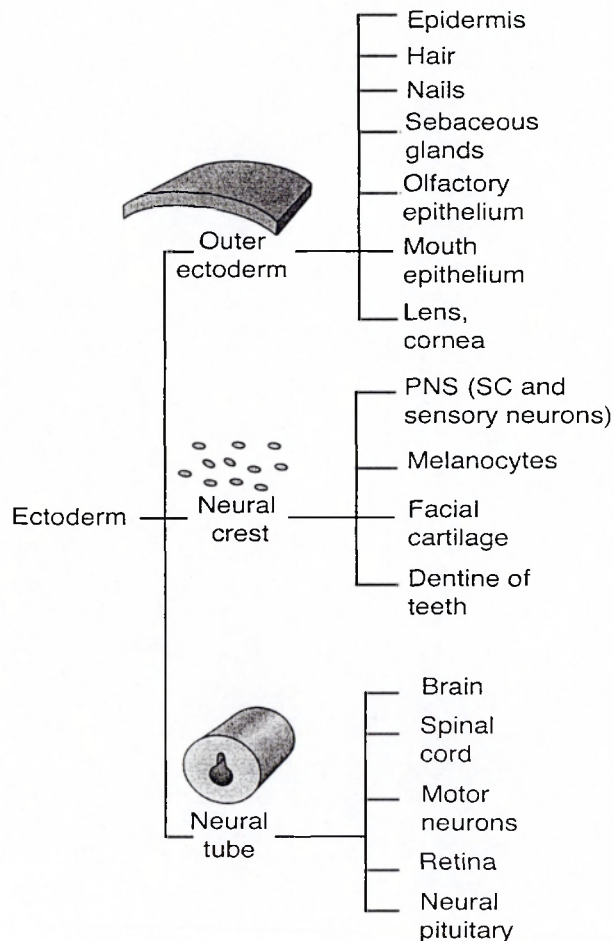


Both the stepwise model and the holoenzyme complex are not sufficient to explain how transcription proceeds in eukaryotes. Recent cytological studies suggest that some co-regulators and components of the general machinery may be segregated from each other in the nucleus (Reyes *et al.*, 1997). It is possible that gene regulator systems have evolved as organised compartments in the nucleus containing increased local concentrations of some co-regulators and a subsets of the transcription machinery. Recently these nuclear compartments were described and they led to the identification of many nuclear structures such as speckles, interchromatin granule clusters, Cajal bodies and PML bodies. These structures have been associated with various transcription factors, co-regulators, RNA polymerases and RNA processing factors (Matera, 1999). Moreover RNA polymerase II is localised in a few discrete foci in isolated nuclei as visualised by immunofluorescence and three-dimensional microscopy (Zeng *et al.*, 1997). Transcriptionally active genes are located preferentially in the center of the nucleus while silenced genes are arranged at the periphery of it (Andrulis *et al.*, 1998; Verschure *et al.*, 1999). In addition to that, other studies established that individual chromosomes are organised into territories that condense and decondense in a cell cycle co-ordinated fashion (Ferreira *et al.*, 1997; Croft *et al.*, 1999). These and other similar regulatory regions may serve to protect and promote an open or accessible chromatin domain, which can be important in regulating cell type specific transcription (Jenuwein *et al.*, 1997).

The model emerging from all these studies is that genes might first undergo an early chromatin rearrangement through initial interactions with primary transcriptional activators and some chromatin remodelling machinery. After those events the DNA is susceptible to the action of enhancer binding factors. In order to completely open the chromatin they could recruit other appropriate remodelling factors, for example histone acetyltransferases. The next step is the co-operative interactions between sequence specific regulators and co-regulators that can initiate promoter binding and subsequent events leading to formation of an active initiation complex. At the end, the disassembly of the initiation complex and the release or the transfer of DNA template from the transcription site to other functional compartments may facilitate RNA elongation and processing.

## 1.5 NERVOUS SYSTEM DEVELOPMENT

The nervous system of vertebrates derives from the ectoderm, one of the three embryonic layers that are established at the gastrula stage, together with mesoderm and endoderm. As gastrulation proceeds the neural fold will form, bend and fuse to form the neural tube overlying the epidermis, and cause delamination of neural crest cells. The neural tube will eventually generate the CNS and its components, neurons, and glial cells, while delamination of the neural crest cells will generate SC and peripheral neurons (Fig. 1.4).



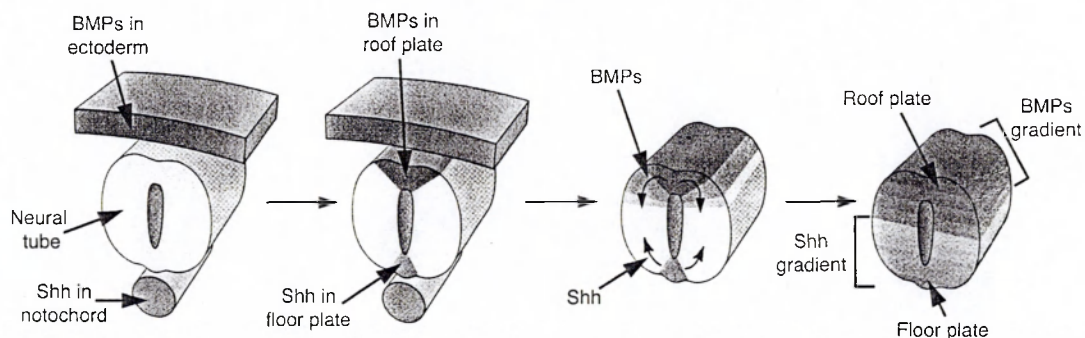
**Figure 1.4**

Schematic diagram of all ectoderm derivatives. The ectoderm is divided into three main regions: the outer ectoderm from which originate the epidermis, the neural tube from which derive CNS components and the neural crest from which derive SC and peripheral neurons. (Modified from Gilbert S.F. *Developmental Biology* 6<sup>th</sup> Edition Sinauer Associated Inc. Publisher, Sunderland, Massachusetts, 2000)

In the generation of the nervous system, the first step consists of the specification of a homogeneous layer of ectodermal cells into the neural plate and the epidermis. Many studies suggest that BMP proteins are involved in this process. In particular BMP-4 and BMP-7 are expressed throughout the gastrula ectoderm except in the future neural plate (Fainsod *et al.*, 1994) and this expression pattern coincides with the presumptive epidermis. Other factors such as *Noggin* and *Chordin* are produced and secreted by the notochord in a graded pattern and are only present in the presumptive neuroectoderm, where BMP-4/7 are absent. Treatment of *Xenopus laevis* animal cap explants with BMP-4/7 promoted the formation of the epidermis, while treatment with *Noggin* and *Chordin* induced the formation of the neural tissues (Wilson *et al.*, 1995; Smith *et al.*, 1992; Sasai *et al.*, 1994). Moreover *Chordin* and *Noggin* can directly inhibit BMP-4/7 by binding to their extracellular domain. Evidence for this model comes from *Drosophila melanogaster*, in which the ablation of the BMP-4 orthologue, *decapentaplegic (dpp)* induces the expansion of the neuroectodermal region (Wharton *et al.*, 1993). On the contrary, ablation of the *Drosophila Chordin* orthologue, *short-gastrulation (sog)*, results in expanded epidermis with less neuroectoderm (Holley *et al.*, 1995). Analysis of these genes in mice revealed that BMP-4 is relevant during gastrulation and formation of posterior body as well as ventral mesoderm (Winnier *et al.*, 1995). Targeted deletion of *Noggin* and *Chordin* separately do not affect ectoderm specification (McMahon *et al.*, 1998). However the double knock out for both genes is embryonically lethal and produces clear defects in forebrain development (Bachiller *et al.*, 2000). Based on all these experiments it seems that factors such as *Chordin* and *Noggin* play an important role in patterning the neural ectoderm into neuroectoderm and neural plate.

The second step requires further patterning of the neural fold along the dorso-ventral (D/V) axis. This patterning is mediated by BMP-4 whose expression is restricted to neural folds. In chicken not only BMP-4/7 are required for this specification, but also a ventralising signal from the notochord is necessary. A good candidate is *Sonic hedgehog (Shh)*, that can change the D/V pattern of pre-existing neural tissue by inducing the expression of *Noggin* in ventral neural plate (Liem *et al.*, 1995; Roelink *et al.*, 1994; Ekker *et al.*, 1995; Hirsinger *et al.*, 1997). Nevertheless the key “player” in directing the D/V axis formation, is the BMP-4/7 gradient present in the

neural tube (Fig. 1.5). Although strong evidence favours this model, other data suggest that BMP-4/7 is not the only factor involved in the process of neural crest specification (Marchant *et al.*, 1998). For instance, expression of basic Fibroblast Growth Factor (bFGF) seems to be required for a correct neural crest induction. It is also possible that bFGF makes the neural crest competent to react to signals such as BMPs (Mayor *et al.*, 1997). Members of the *Wingless* family of growth factors (Wnt) Wnt1/3A have also been implicated in neural fold induction through cooperation with BMP-4/7 or bFGF (Saint-Jeannet *et al.*, 1997). All these data indicate that induction of the neural crest fate might be determined by the position of a cell in a network of morphogen gradients, such as BMP-4/7, bFGF, Wnt-1/3A, along each axis in the developing embryo (Panchision, 1998).



**Figure 1.5**

Specification of the nervous system components. The BMP proteins are first expressed in the outer ectoderm and then in the roof plate. These proteins are responsible for the formation of the epidermis and for the specification of the dorso-ventral axis. The other component essential for the formation of the dorso-ventral axis is *Shh*, which is expressed in the floor plate. *Shh* is responsible of the formation of the ventral neural tube and it is involved in the generation of motor neurons and oligodendrocytes. See text for details. (Modified from Gilbert S.F. Developmental Biology 6<sup>th</sup> Edition Sinauer Associated Inc. Publisher, Sunderland, Massachusetts, 2000)

## CNS DEVELOPMENT

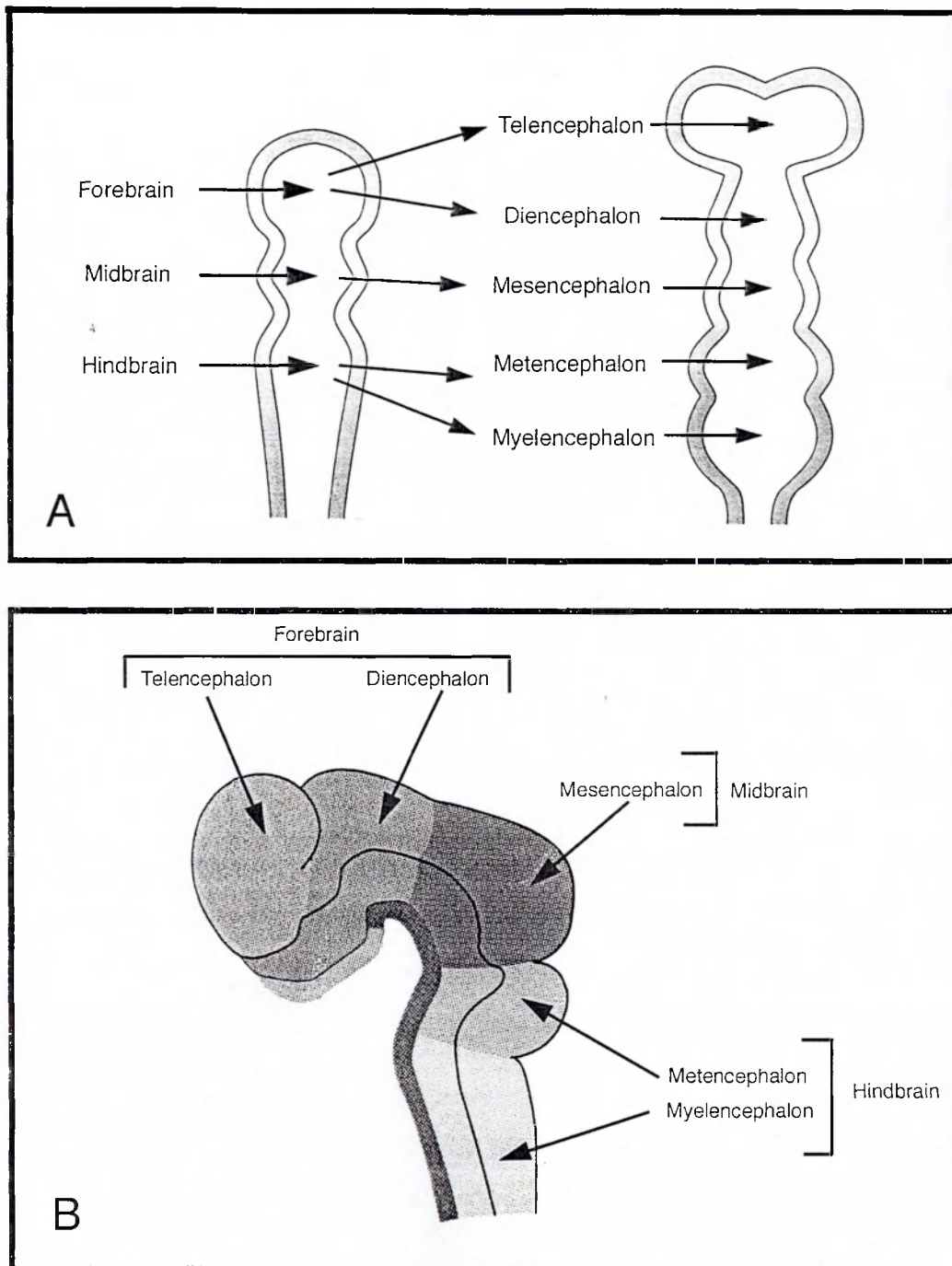
The differentiation of the neural tube into various regions of the CNS occurs simultaneously in three different ways:

1. at the anatomical level with the constriction of its lumen to form the chambers of the brain and the spinal cord;
2. at the tissue level with the rearrangement of the cell populations within the wall of the neural tube to form the brain and the spinal cord;
3. at the cellular level, with the differentiation of the neuroepithelial cells into neurons and glial cells.

At the tissue level the most anterior part of the tube develops into the three primary vesicles: forebrain (*prosencephalon*), midbrain (*mesencephalon*), and hindbrain (*rhombencephalon*) (Fig. 1.6). The forebrain is subdivided into the anterior telencephalon and the more caudal diencephalon. The telencephalon then gives rise to the cerebral hemispheres, while the diencephalon will form the thalamic and the hypothalamic brain regions. The midbrain is not subdivided into segments; instead the pattern of cells is controlled by a long-range action of many signals, the principal of which are FGF8 and Wnt molecules. The hindbrain is subdivided into a posterior myelencephalon and a more anterior metencephalon, from which originates the cerebellum. It is also divided in smaller compartments called *rhombomeres* that form clusters of neuronal cell bodies, the ganglia. Cells of a rhombomere can mix within, but not between adjacent rhombomeres (Guthrie and Lumsden, 1991).

It is possible to follow CNS organisation at the cellular level because the neural tube is polarised along its D/V axis. As discussed above, this polarity is induced by signals coming from its immediate environment (Fig. 1.5). The epidermis imposes the dorsal pattern, while the notochord induces the ventral pattern. The BMP-4/7 gradient causes the expression of different types of transcription factors in cells at various distances from the roof plate, giving them a specific identity. The ventral tube is specified by external cues, and in particular by *Shh* and *retinoic acid*. *Shh* is initially produced by the notochord, where it is cleaved and secreted. The secreted form is the active part of the protein, which induces the formation of the floor plate of the neural tube. The floor plate cells also secrete *Shh* which forms a gradient highest at the most ventral portion of the neural tube (Roelink *et al.*, 1995). This gradient specifies the type

of neurons that are created (Briscoe *et al.*, 1999). The variety of Shh concentrations induces the expression of a different set of cell-fate transcription factors and they determine the destiny not only of neurons but also of OL. Shh is also a repressor of genes that otherwise will be expressed throughout the neural tube causing a dorsalization of the phenotype (Echelard *et al.*, 1993; Roelink *et al.*, 1994).



**Figure 1.6**

Schematic diagram of the CNS vesicles (A). The more anterior part of the CNS, the forebrain is sub-divided into the telencephalon and the diencephalon, whereas the hindbrain is sub-divided into the metencephalon and the myelencephalon (B). (Modified from Gilbert S.F. *Developmental Biology* 6<sup>th</sup> Edition Sinauer Associated Inc. Publisher, Sunderland, Massachusetts, 2000).



## OLIGODENDROCYTE DEVELOPMENT

The developmental pattern of OL, is best understood in spinal cord and in optic nerve, while the origin in the forebrain is less well known. The initial specification of Oligodendroglial Precursors Cell (OPC) depends on signals coming from the notochord and the ventral roof plate. OPC originate in a region adjacent to the floor plate in the same zone of the neural tube that gives rise to motor neurons (Noll and Miller, 1993).

A critical signal in OPC specification is Shh and the basic Helix-Loop-Helix (bHLH) genes *Olig1* and *Olig2*. After their appearance, OPC migrate throughout the neuraxis. A precocious marker that identifies these cells is recognized by the A2B5 antibody, directed against cell surface gangliosides, which stains bipolar and mobile cells (Fig. 1.7). When cultured in vitro, these cells are responsive to both basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (Noble *et al.*, 1988). These cells mature into pro-oligodendroblasts that are characterised by a reduced motility and the expression of a fine arbor of processes. They also express specific sulfatides that can be labelled with the O4 antibody. In vitro, these cells proliferate primarily in response to bFGF (Pfeiffer *et al.*, 1993). Only after this stage of development, the cells exit the cell cycle and start to express galactocerebroside (GalC), a major glycolipid of myelin (Raff *et al.*, 1978). These cells are identified as O1 cells. The last steps in OL development require a transition from pre-myelinating to myelinating OL. These last changes are not well understood, but axonally derived signals, like neuregulins (NRG), seem to be required (Fernandez *et al.*, 2000).

Recent studies have shown that PDGFR $\alpha$ <sup>+</sup> OL develop also from region of the neuroepithelium that generates somatic motor neurons, suggesting the possibility of a common progenitor for these two cell types (Richardson, 2001). The existence of this common progenitor between neuron and OL is supported by clonal analysis performed in embryonic chick spinal cord (Leber and Sanes, 1995).

### *Sonic Hedgehog*

A recent study showed that regions of the telencephalon, where Shh is expressed, are the first to express early OL markers (Nery *et al.*, 2001). Grafting and neural plate explant studies showed that one of the main agents involved in the initial generation of OL is Shh. It induces oligodendrogenesis and is required for OPC



specification in chick embryo spinal cord (Orentas *et al.*, 1999). In the spinal cord, OPC differentiate from bipolar migratory precursor cells that arise from specific regions of the ventral neuroepithelium. Then they disperse through the developing gray matter to populate white matter tracts. These precursor cells give rise to progenitors that migrate to their correct location where they first proliferate and then terminally differentiate to OL.

#### *Platelet-Derived Growth Factor Receptor $\alpha$ (PDGFR $\alpha$ )*

A marker for OPC differentiation is PDGFR $\alpha$ . Both mRNA and protein were detected in embryonic spinal cord at E12.5 in mouse (E14 in rat) (Fig. 1.7). By E17 in mouse, PDGFR $\alpha$  expressing cells are distributed throughout the spinal cord (Pringle *et al.*, 1996). When these cells are purified from embryonic rat spinal cord at E16 and put in culture, they differentiate into OL (Hall *et al.*, 1996). Recently the analysis of the PDGF-A deficient mice showed a massive decrease in the amount of OL in the brain. Nevertheless there are other regions in the brain of these animals in which OL mature normally (Fruttiger *et al.*, 1999).

#### *PLP/DM-20*

Another marker of the early steps in OL development is the myelin gene *plp/dm-20*. In mice early *plp/dm-20* expressing cells are detected in the ventricular zone before E10.5 (Fig. 1.7). Between the time of emergence and birth the number of *plp/dm-20*<sup>+</sup> cells increase and they progressively invade the future white matter tracts (Timsit *et al.*, 1995). The period during embryonic development in which these precursors are first detected matches the emergence of the first axonal tracts. Although *plp/dm-20* and PDGFR $\alpha$  cells were both localised in the same region of the embryonic spinal cord, they are never co-localised in the same cell at early stages. It is likely that *plp/dm-20* and PDGFR $\alpha$  represent two distinct populations of OL. Moreover *plp/dm-20*<sup>+</sup> cells are not only differently located from those expressing PDGFR $\alpha$ , but when put in culture they are not responsive to PDGF (Spassky *et al.*, 1998).

## TRANSCRIPTIONAL CONTROL IN OLIGODENDROCYTE DEVELOPMENT

Recently some transcription factors involved in specifying OL development have been identified *in vivo*.

*Nkx2.2*

*Nkx2.2* is a homeodomain transcription factor that is known to be involved in the regionalization of the ventral zone. In particular it is known to be involved in the generation of the motor neurons.

Recent studies suggest that, both in chicken and in rodent spinal cord, OL originate from the ventral zone pattern of the neural tube that is *Nkx2.2* positive (Xu *et al.*, 2000; Qi *et al.*, 2001). Double labelling experiments showed a co-localisation between *Nkx2.2* and some OL markers. The importance of *Nkx2.2* in directing oligodendroglialogenesis is shown also by the analyses of *Nkx2.2* null mice. In these mice there is a strong reduction in the number of OL that can differentiate, while the number of OPC that express both *PDGFR $\alpha$*  and *Olig1/Olig2* genes is increased.

*Sox-10 (Sry-box containing)*

*Sox-10* is a member of superfamily of High-Mobility-Group (HMG) protein. Members of this family bind to the consensus sequence (A/T)(A/T)CAA(A/T) in the minor groove of DNA, strongly bending it (Wegner, 1999). *Sox-10* transcripts can be detected in mouse embryo from E8.5 in the dorsal neural tube and in the presumptive neural crest region (Fig. 1.7). The expression of *Sox-10* disappears in CNS until day E13.5 where it reappears. The next day, *Sox-10* positive cells are detected in the spinal cord correlates with the first appearance of OPC. From this day of development, cells positive for *Sox-10* are detected in all regions of the brain. Later in development and in the adult, *Sox-10* is preferentially found in OL. Moreover in the CNS high expression of *Sox-10* is recovered in areas with myelinated fibers (Kuhlbrodt *et al.*, 1998). A recent paper also shows that *Sox-10* expression is restricted to myelin forming oligodendroglia (Stolt *et al.*, 2002). In the same study the authors showed that in *Sox-10* deficient mice OPC precursors are generated, however the terminal differentiation of OL is impaired. The relevance of *Sox-10* in OL development is underlined also by the fact that its expression is detected at about the same time of *Olig2* and precedes that of

*Olig1* in the same cells. Ectopic expression of *Olig* genes in brain causes ectopic expression of Sox-10 in vivo (Zhou *et al.*, 2000). Moreover classical in vitro transcriptional studies, showed that Sox-10 directly controls the expression of both Myelin Basic Protein (MBP) and Proteolipid Protein (PLP) suggesting that it is a general regulator of myelin genes expression (Stolt *et al.*, 2002).

### *Olig1 and Olig2*

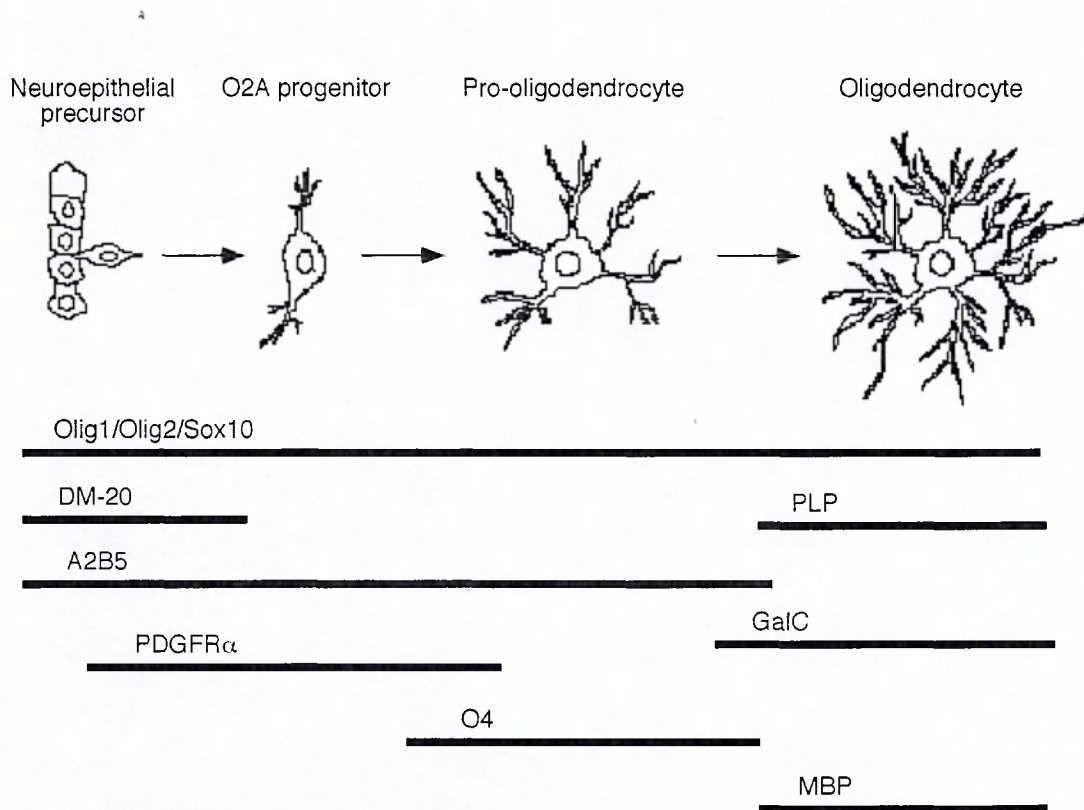
Recently two closely related bHLH genes, named *Olig1* and *Olig2*, preferentially expressed in OL cell lineage, were identified (Lu *et al.*, 2000; Zhou *et al.*, 2000). They represent the first known transcription factors suggested to be fate determining genes for OL. Their sequence is almost identical in the bHLH domain, while it is completely divergent in the loop region. At the structural level, the bHLH domain resembles that of other bHLH genes, such as *NeuroD* and *Neurogenin*, which are known to be fate specific genes in neuronal development. In the ventral spinal cord they are both expressed at E9.5 (Fig. 1.7). At this stage *Olig2* is more evident than *Olig1*. At E10.5 both genes are expressed in the ventricular zone. No expression was found in the DRG of the PNS. In adult rat CNS, the expression of both genes is restricted to regions enriched in OL.

In situ hybridisation studies showed that *Olig* gene expression precedes that of both *plp/dm-20* and *PDGFR $\alpha$* . *PDGFR $\alpha$*  and *Olig1* are co-expressed in the same cell types. Lu and co-workers (Lu *et al.*, 2000) showed that *Shh* is both necessary and sufficient for normal *Olig* expression in the developing CNS. In fact, ectopic induction of *Shh* causes an ectopic expression of *Olig* genes. Moreover mRNA for both *Olig* genes is not detected in embryos homozygous null for *Shh*, either in brain and in spinal cord. On the other hand Zhou et al (Zhou *et al.*, 2000) showed that ectopic expression of *Olig2*, induces the expression of Sox-10 in vivo, but they do not detect the expression of myelin genes indicating that other factors are necessary for full development of oligodendrocytes.

### *Id2*

In vitro analysis of OL differentiation led the identification of other basic bHLH genes involved in their differentiation. They are mainly involved in timing the

differentiation of OL. Those transcription factors include at least one member of the Id family, *Id2*, whose overexpression blocks OL differentiation and enhances proliferation (Wang *et al.*, 2001).



**Figure 1.7**

Schematic representation of oligodendrocyte development and markers expressed at each step. See text for details.

*PNS DEVELOPMENT*

The different components that make up the PNS originate from different germ layers. During embryonic development neurons and their associated glial cells derive from the neuroectoderm, while the nerve sheath and nerve vasculature derive from the mesoderm. The cells that give rise to neurons and glial cells are the neural crest cells. Neural crest cells are formed at the border between the neural plate and the presumptive epidermis, overlaying the lateral plate mesoderm in a gastrulating embryo.

Two principal steps are involved in determining their development: neural crest delamination and neural crest fate determination.

Neural crest delamination is important in determining subsequent differentiation and migration of these cells towards target organs. At the beginning they have to switch from an epithelial to a mesenchymal morphology. In *Xenopus*, chicken and mouse, neural crest induction is marked by the expression of the zinc-finger protein *Slug*. In chick and mouse embryos, cells of the dorsal midline express *Slug* before delamination. Although *Slug* has proven to be a useful marker for presumptive neural crest cells, its involvement in neural crest induction and delamination remain unclear, in fact its deletion does not cause any failure in this process (Jiang *et al.*, 1998). Delamination of neural crest cells starts at the rostral end of the embryo in *Slug* positive migrating cells. It was also demonstrated that ectopic expression of *Noggin* can prevent migration and since *Noggin* is a BMP-4 inhibitor it is possible that somehow it positively regulates the epithelial-mesenchymal transition (Sela-Donenfeld *et al.*, 1999).

A series of homotypic graft experiments demonstrate that neural crest, originating from rostral to caudal levels, can generate distinct but also overlapping sets of derivatives (Baker *et al.*, 1997). For example only a small population of trunk neural crest generate chromaffin cells of the adrenal, while SC derive from neural crest cells of the entire antero-posterior axis (Le Douarin *et al.*, 1993). Many tracing experiments were performed to understand if the environment is instructive on a homogeneous population of neural crest cells or selective on a collection of committed cells. These studies show that while most pre-migratory cells, such as sensory neurons and glia, are multipotent (Frank and Sanes, 1991), there are also precursors generating a single unique neural crest derivative. To understand the entire repertoire of these cells, rat and mouse neural crest cells were grown at clonal density. These studies showed that many

cells, before leaving the neural crest, are multipotent self renewing stem-like cells (Ito, 1993). These clonal cell cultures allowed also the identification of factors that are potentially involved in the lineage determination of the neural crest. These factors act in an instructive manner since they promote development of one lineage. One of these factors is GGF-2. This is the product of the neuregulin gene and it can induce differentiation of neural crest cells in SC (Shah *et al.*, 1994). On the contrary, BMP-4 or BMP-2 promote neuronal differentiation, while smooth muscle cells are induced by TGF $\beta$  (Shah *et al.*, 1996). The importance of GGF-2 is shown also in vivo and given its importance in determining SC development I will discuss it in the next session.

### *SCHWANN CELL DEVELOPMENT*

During vertebrate development, SC originate from a multipotent migratory cell population that derives from the neural crest. In this process, cells migrate from the dorsal ridges of the neural folds to form the migratory population known as neural crest cells.

The process of SC development involves three transitions (Fig. 1.8):

1. from neural crest cells to precursor SC;
2. from precursor to immature SC;
3. from immature to the two different types of adult SC, the myelinating and the non myelinating SC.

SC precursors are identified in rat nerves at E14-E15 (E12-E13 in mouse), while the immature SC are present from E17 to birth in rat (E15 in mouse) (Bhattacharyya *et al.*, 1991; Lee *et al.*, 1997; Zhang S.M. *et al.*, 1995). The first contact between early post migratory neural crest and axons probably occurs when both start to migrate away from the neural tube through the rostral part of the somites. This migration along the rostral somite is mediated by interaction with attractive cues such as tenascin, laminin and thrombospondin and repulsive cues, such as ephrins and their receptors, F-spondin, cytotactin binding proteoglycan, cadherin and versican (Krull *et al.*, 1998). These signals are responsible for PNS segmentation; in fact they direct axons and the associated neural crest cells towards target organs. All these transitions can be distinguished in SC based on their morphology. The intimate relationship between neural crest, its derivatives and axons are a prerequisite for these transitions. To

investigate how neural crest cells become SC, a good model is represented by developing rat sciatic nerve. In fact approximately two days after neural crest migration, between E13 and E14, the axons project out into the rat hind limb. At this stage glial cells are strictly associated with the axons (Jessen *et al.*, 1994).

#### *Neural crest – SC precursor transition*

One of the major problems in defining the development of SC from neural crest cells is the absence of early differentiation markers. Precursor SC have sheath like processes, which contact each other and encircle groups of axons into large bundle, a phenomenon that in rat sciatic nerve is present at E14 (Fig. 1.8). SC precursors are characterised by some differentiation markers, such as p75, Growth associated Protein-43 (GAP-43), Neural Cell Adhesion Molecule (N-CAM) and L1 adhesion molecule. Another precocious marker identified in a population of neural crest cells is the P0 mRNA. During development it is expressed at very low level, in precursor and immature SC regardless of whether they are destined to attain myelinated or non-myelinated phenotypes (Bhattacharyya *et al.*, 1991; Lee *et al.*, 1997). SC precursors need an axonally derived signal to survive. In fact if they are dissociated from the neurons and they are put in culture, they die (see chapter 5: SC-axon reciprocal influences). The signal involved in survival of SC precursors is  $\beta$ -Neuregulin (NRG) (Mirsky and Jessen, 1999).

#### *Neuregulins and ErbB receptors*

Neuregulins play fundamental roles in cell survival, migration and differentiation of many cell types. There are four genes that encode neuregulins: *NRG-1*, *NRG-2*, *NRG-3* and *NRG-4*. Little is known of the role of *NRG-2*, *NRG-3* and *NRG-4*, whereas *NRG-1* has been most intensely studied and it is fundamental for SC and OL survival and differentiation. Neuregulins are a family of growth factors, characterised by the combination of different domains: an Epidermal Growth Factor (EGF)-like motif, a signal peptide, an immunoglobulin (Ig)-like domain, a cysteine-containing N-terminal domain, a glycosylation domain and a transmembrane domain. All *NRG-1* isoforms can be proteolytically cleaved and released from the cell surface.

Alternative splicing of the *NRG-1* gene produces many variants, which are grouped in three different isotypes. Members of isotype 1 include NDF and Acetylcholine Receptor Inducing Activity (ARIA), members of isotype 2 include GGF-2 and members of group 3 include Sensory and Motor Derived Factor (SMDF).

Targeted deletion of *NRG-1* in mice results in an early embryonic lethal phenotype, caused by cardiac defects. However, just before the embryo dies it is possible to show the almost complete absence of SC precursors (Meyer and Birchmeier, 1995). In vitro studies showed that NRG-1 is important to drive the differentiation of neural crest progenitors toward a glial cell fate. In the early post-natal peripheral nerve, NRG-1 is an important survival factor and it regulates the number of pre-myelinating SC (Grinspan *et al.*, 1996). The level of NRG-1 protein in adult mice is strongly reduced as compared to the early postnatal period. Moreover a recent paper showed that addition of GGF-2 to SC-DRG cocultures blocks the myelination process and it causes demyelination if added to co-cultures that have already myelinated (Zanazzi *et al.*, 2001).

The role of NRGs in the CNS is not well understood. In OL, NRG-1 promotes proliferation of OPC and in long term cultures it constitutes a potent survival factor (Fernandez *et al.*, 2000). In vivo NRG inhibitors reduce the OPC number in optic nerve. It seems to be required for oligodendroglioneogenesis; in fact spinal cord explants of *NRG-1* null mice, do not develop OPC until they are rescued by external addition of recombinant NRG-1 (Vartanian *et al.*, 1999).

Receptors for these ligands are the four members of the EGF family of receptor tyrosine kinases: ErbB1 (EGF-receptor), ErbB2, ErbB3 and ErbB4. The ErbB receptors contain a large extracellular ligand-binding domain, a single transmembrane domain and an intracellular part with a COOH-tail and a tyrosine kinase domains. They usually form heterodimers on the cell surface, and nearly all combinations are possible. This mechanism leads to receptor phosphorylation and activation of downstream signalling pathways. However, ErbB2 has no affinity for NRGs, while ErbB3 receptor lacks the tyrosine kinase activity. The lack of the catalytic activity of the ErbB3 receptor is overcome by the process of ligand induced dimerization, in which one of the other ErbB members is recruited as co-receptor.



Many of these receptors are expressed in neurons and in glial cells, both OL and SC, during development (Burden and Yarden, 1997). The ErbB2/ErbB3 heterodimer is the primary NRG-1 receptor in SC, while in OL all receptors are expressed (Canoll *et al.*, 1996).

Knockout mice for *ErbB3* cause death soon after birth, and their nerves are devoid of SC, presumably because they lose SC precursors even if axons can reach their normal target with normal innervation (Riethmacher *et al.*, 1997). The influence of SC on axons and the role of these null mice in understanding the importance of SC signalling to axons I will present later (see chapter 5: SC-axon reciprocal influences). Like  $\beta$ -NRG null mice, mice lacking ErbB2 receptor die for cardiac defects at an embryonic stage in which it is not possible to evaluate the effect on SC development except in ventral roots (Lee *et al.*, 1995; Garratt *et al.*, 2000). Recently transgenic mice that can rescue this cardiac defect were generated (Morris *et al.*, 1999; Woldeyesus *et al.*, 1999). In both studies, rescued animals survived until birth, but all peripheral nerves were devoid of SC, showing a phenotype similar to the one described for ErbB3 knock out. The ablation of the *ErbB4* gene results in precocious embryonic death, but the fact that it is not expressed in SC makes it not relevant in regulating the development of SC precursors (Gassman *et al.*, 1995; Grinspan *et al.*, 1996).

In the CNS targeted disruption of *ErbB2* shows that it is not required for the formation of early OL lineage, while it seems to be involved in later stages of OL development (Park *et al.*, 2001).

#### *Precursor – immature SC transition*

This transition occurs between E14 and E17 in rat and in vivo it is characterised by a series of morphological changes (Fig. 1.8). Immature SC invade bundles of axons and sort them into smaller groups. Depending on the size of axons, they associate with only one large axon or with multiple small caliber axons, in order to adopt a pro-myelinating or a non pro-myelinating phenotype, respectively. When put in culture immature SC are not very mobile and they have a bi- or tri-polar morphology, whereas precursor SC have high mobility and they are flattened. Immature SC are characterised by the expression of specific markers such as S100 and the sulphatide antigen O4 (Jessen and Mirsky, 1991). These cells can survive when cultured at high density in a

defined medium without external addition of NRG or DRG conditioned medium (Jessen *et al.*, 1994). In vivo analysis of SC development revealed that the switch from axon-dependent to axon-independent survival occurs gradually as SC mature in peripheral nerves (Fig. 1.8).

#### *Immature to non-myelinating SC transition*

Non-myelinating SC express several characteristic surface markers such as p75, Glial Fibrillary Acidic Protein (GFAP), N-CAM, L1. These markers are also present in pro-non-myelinating SC. The analysis of *L1* knockout mice revealed that only non-myelinating SC of the sensory fibers are affected and there is a reduced association between sensory axons and non-myelinating SC (Dahme *et al.*, 1997). Transplantation experiments between wild type SC and *L1* deficient mice showed that these SC in contact with affected axons reproduced the *L1* deficient phenotype, strongly suggesting that *L1* expression on axons is essential for the formation of unmyelinated fibers (Haney *et al.*, 1999). Probably heterophilic interactions between *L1* on the axonal membrane and other components on the non myelinating SC membrane are required for the formation of these fibers.

#### *SC-axon reciprocal influences*

During PNS development, both SC and axons play a central role influencing each other in their different functions. In fact while it was well known from many studies that axons directly regulate SC development, it is now becoming evident that even SC can directly influence neuronal survival and development.

Signals coming from axons are determinants for the survival of SC precursors. When these cells are dissociated from neurons and put in culture, they undergo programmed cell death, strongly suggesting that SC precursors need an axonally derived signal to survive. In fact both close proximity in culture to DRG neurons and a conditioned medium prepared from P1 DRG can rescue the programmed cell death of SC precursors (Dong *et al.*, 1995). These studies showed that a soluble hybrid protein containing the extracellular domain of ErbB4 receptor blocks this activity. In addition to that, when NRG is added to the medium it mimics the effect of the neuron conditioned medium, supporting the survival of SC precursors in vitro (Mirsky and

Jessen, 1999). In SC development, the important role of NRG is suggested by the fact that its mRNA is present at the right time and place to act as a neuron-instructive signal. It is in fact highly expressed in DRG and ventral horn in motor neurons at E14 (Bermingham-McDonogh *et al.*, 1997; Marchionni *et al.*, 1993). Studies on the processes that govern SC development showed that the switch from axon-dependent to axon-independent survival is likely to occur gradually as SC develop from precursors and mature in peripheral nerves (Fig. 1.8). This is demonstrated by the fact that when SC-axonal contact is lost after nerve transection at perinatal stages, there is a significant SC death that can be prevented by the addition of NRG. On the contrary if the same transection is performed in an animal 15 days after birth there is very low apoptotic death showing that SC are already able to survive (Trachtenberg and Thompson, 1996; Syroid *et al.*, 1996). This mechanism of number control during SC maturation is relevant in vivo to exactly regulate peripheral nerve development. In fact the dependence of precursors on axonal contact is probably involved in matching precursor numbers to axon numbers (Zorick *et al.*, 1999).

Axons directly influence the decision of a SC to become myelin-forming or not. In fact axon diameter determines if an immature SC will acquire a myelinated or a non-myelinated phenotype. The decision to become a myelinating versus non-myelinating SC is determined by cell-extrinsic signals coming from the axons. The identity of the axonal signals responsible for this choice is not known, although it is determined by the axonal diameter; in fact only axons with a diameter bigger than 1  $\mu\text{m}$  will become myelinated, whereas smaller axons will not. However SC directly control the number of neurofilaments of the axons and their phosphorylation state. This is particularly important because a change in neurofilaments number is important in determining axon diameter (Martini, 2001).

Nevertheless, it is becoming evident that not only are axons influencing SC development, but also SC exert fundamental effects on neuronal final development. SC play a role in controlling axonal survival, regeneration and normal development. They also somehow affect changes in the axons in some inherited human neuropathies.

SC directly support axonal survival as evidenced by the analysis of the ErbB3 knock out mice. These mice die soon after birth, but their nerves are devoid of SC, presumably because they lose SC precursors even if axons can reach their normal target

with normal innervation (Riethmacher *et al.*, 1997). This suggests that cells of the SC lineage are not necessary for normal axonal development; however analysis of DRG survival in these mice revealed that 80% of them die before reaching the final target between E13 and E18, indicating that sensory neuron death is due to lack of SC and their precursors. These studies underline that during PNS development a cross talk between SC precursors and neurons is necessary to reach a normal development at least in DRGs.

Signalling from SC to neurons is also relevant after axonal damage. This process known as Wallerian degeneration, causes a retraction of proximal and distal stumps of the nerve near the lesioned point. In correspondence to all these defects SC de-differentiate to an immature phenotype. At this stage SC proliferate and they form endoneurial tubes, called band of Bungner. The basal lamina produced by these SC form a trace in which axons can regenerate. After re-establishment of contact between SC and axons these SC stop proliferating.

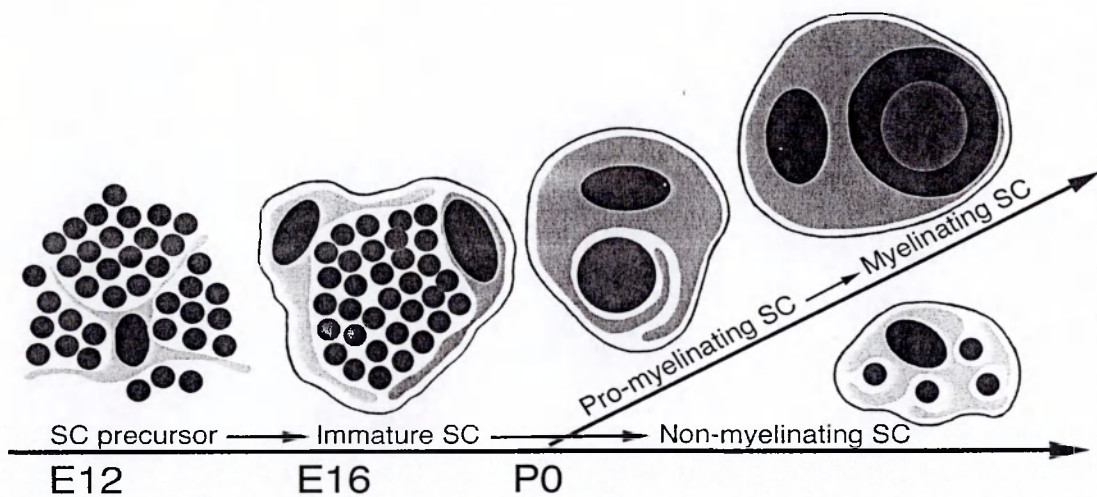
SC also influence the formation of the protective connective tissue layers that surround boundles of nerve fibers as shown by the analysis of *Dhh* null mice (Parmantier *et al.*, 1999), and as we discussed in the description of the perineurium in Chapter 3.

SC mediates also the spacing of  $\text{Na}^+$  channel clusters during development of the Node of Ranvier (Salzer, 2002).  $\text{Na}^+$  channels are positioned in the middle of the Node of Ranvier and their distribution is particularly important for the saltatory conduction of impulses. Recent papers showed that the targeting of these channels at the Node of Ranvier is regulated by myelinating glia not only in the PNS but also in the CNS (Boiko *et al.*, 2001). Other papers suggests that soluble factors could be involved in the clustering of these channels at the Node of Ranvier (Kaplan *et al.*, 1997; Martini, 2001).

More importantly it has been shown that some of the mutations that cause human inherited neuropathies such as Charcot Marie Tooth (CMT) disease, Dejerine Sottas Syndrome (DSS) and Congenital Hypomyelination (CH) disease are due to a primary defect in SC. However, as a consequence of these mutations, some patients show also changes in the axons, such as reduced axonal caliber and alteration in the phosphorylation state of neurofilaments (Bjartmar *et al.*, 1999). These secondary

defects affecting the axons could lead to axonal degeneration with subsequent severe clinical consequences (Sahenk Z., 1999).

All these observations strongly suggest the existence of a strict relationship between SC and axons. Both axons and SC cooperate for development and maintenance of the PNS.



**Figure 1.8**

Schematic representation of the main transitions occurring during SC development in mice: from neural crest cell to precursor cell; from precursor to immature Schwann cell and from immature to myelinating or non-myelinating Schwann cell (Modified from Jessen and Mirsky, TINS 22(9), 1999).

### TRANSCRIPTIONAL CONTROL IN SCHWANN CELL DEVELOPMENT

The differentiation of SC during development is controlled by many transcription factors (Fig. 1.9).

#### *Pax-3*

Pax-3 is a member of the murine Paired Box gene family composed of at least nine members in vertebrates (Mansouri *et al.*, 1996). The *Pax* genes contain a DNA binding domain homologous to the pair rule gene *paired* in *Drosophila*. Several members of this family contain also an homeobox domain. The Pax-3 recognition site contains two DNA sequence blocks, encompassing 18bp: an ATTA motif and a GTTCC region. In the mouse Pax-3 expression starts at E8.5 in the dorsal neuroepithelium and by E 9.5 is present in somitic mesoderm and neural crest. In SC lineage Pax-3 mRNA is expressed in the embryonic sciatic nerve from E12 and peaks at E17 (Blanchard *et al.*, 1996). Pax-3 level of expression falls gradually after birth and reaches the lowest level 3 days after birth. It is also expressed by E14.5 in immature or precursor SC and strictly in non-myelinating SC in sciatic nerves 5 days after birth and in adult animals. All these data indicate that Pax-3 is associated with neural crest and non-myelinating SC (Kioussi *et al.*, 1995). Nerve crush and transection experiments showed that Pax-3 expression is dependent on axonal contact. These results suggest that Pax-3 is involved in determining the fate of immature SC into a non-myelinating phenotype. The role of Pax-3 in SC development has been studied also in two natural mutant mice: *spotched* (*sp*) and *spotched delayed* (*sp<sup>d</sup>*). In the homozygous state both mice are embryonically lethal. The *sp* mouse dies at E13.5 and its nerves lack SC. The *sp<sup>d</sup>* mouse die at E18.5 and it has a small number of SC at E13.5 but at E15.5 SC could no be longer detected. These studies suggest that neural crest delamination or SC precursors are affected in these mice or that SC precursors can form, but they can no longer survive.

#### *Sox-10*

Another transcription factor involved in specifying SC development is Sox-10. In the PNS, Sox-10 can be detected in migrating mouse neural crest cells after E8.5 (Fig. 1.9). In later stages of embryonic development, Sox-10 is detectable in enteric

ganglia, glial cells of the sensory and sympathetic ganglia and from E 10.5 also in SC precursors. Its expression remains high in enteric glia and in SC during all subsequent stages of embryonic and postnatal development. The natural *Sox-10* mutant, *Dominant megacolon (Dom)*, has many defects in all neural crest derived tissues. Depending on genetic background, mice homozygous for this mutation die between E13 or immediately after birth, however in these mice no SC alterations have been reported. The targeted *Sox-10* ablation in mice shows that in the heterozygous state the animal resemble the same phenotype observed in the spontaneous *Sox-10 Dom* mutant. More interestingly homozygous *Sox-10* null mice do not develop SC or satellite cells, even if sensory neurons are normally formed in their DRGs (Britsch *et al*, 2001). However at later developmental stages these animals have severe degeneration of both sensory and motor neurons, resembling the same phenotype observed in *ErbB3* null mice. The analysis of expression of these genes in *Sox-10* null mouse, revealed that *Sox-10* controls *ErbB3* expression in neural crest cells (Britsch *et al*, 2001).

Co-transfection experiments showed that *Sox-10* synergistically interacts with *Pax-3* and the POU domain transcription factor *Oct-6* to activate transcription of a reporter gene in which DNA binding sites for each of these factors were cloned. On the contrary it represses the transcriptional activity of the zinc finger protein *Krox-20* (Kuhlbrodt *et al.*, 1998). These in vitro results suggest that *Sox-10* may be relevant in SC development.

#### *Oct-6/SCIP/Tst1*

Another transcription factor involved in the regulation of SC development is *Oct-6/SCIP/Tst1*. It belongs to the class III family of POU domain transcription factors. This family consists of two conserved regions, an amino terminal specific domain and a carboxy terminal homeodomain. The consensus-binding site for these transcription factors is ATTTGCAT; the homeodomain binds to the ATTT sequence, while the specific domain recognises the GCAT region (Verrijzer *et al.*, 1992). During early stages of development *Oct-6* is expressed in many ectodermally derived tissues. In later stages it is expressed in proliferating OL precursors and in some neurons of the CNS (Collarini *et al.*, 1992). In the SC lineage both *Oct-6* mRNA and protein can be detected in SC precursors, rising to a peak shortly after birth (Fig. 1.9). In adult it is

expressed at low levels in non-myelinating SC (Arroyo *et al.*, 1998; Blanchard *et al.*, 1996). In *Oct-6* null mice SC achieve a 1:1 relationship with axons but animals die at birth from respiratory defects (Bermingham *et al.*, 1996). However a small number of knock out mice can survive, and these animals show a delayed myelination (Jaegle *et al.*, 1996). An explanation for this rescue is that Oct-6 might be involved in determining the transition from pro-myelinating to myelinating SC. It is also possible that other POU genes compensate for its absence. Other in vitro and in vivo studies, using a dominant negative form of Oct-6, postulated that it can act as a negative transcriptional regulator and a general repressor for myelin genes (Monuki *et al.*, 1993; Weinstein *et al.*, 1995). However a recent paper showed that Oct-6 is a direct Krox-20 activator in the “so-called” dominant negative transgenic mice. In particular the authors showed that it acts as a positive regulator at the beginning of the myelination process (Ghislain *et al.*, 2002). Thus, Oct-6 probably serves to regulate the timing of myelination by regulating how SC transit from pre-myelinating to pro-myelinating to myelin-forming.

#### *Krox-20 and Krox-24*

Egr-1 (Krox-24) and Egr-2 (Krox-20), two zinc finger transcription factors belonging to the Early Growth Response (Egr) family, are expressed in the SC lineage. This family includes also *Egr-3* and *Egr-4* genes. In addition to a DNA binding domain, both Krox-20 and Krox-24 contain an activation domain and a domain that is able to interact with two co-factors NAB1 and NAB2 (NGF-1A-binding protein) (Russo *et al.*, 1995; Svaren *et al.*, 1996). Both genes are activated around E10.5 in the peripheral nerve in a non-overlapping pattern. Only Krox-24 is expressed in SC precursors while both genes are present in the dorsal and ventral roots close to the neural tube. Krox-20 is activated in the peripheral nerve around E15, exactly in the same period in which Krox-24 is downregulated (Fig. 1.9). The transition between Krox-20 and Krox-24 expression is thought to be associated with the acquisition of a one-to-one relationship between SC and axons. Krox-24 expression is strongly re-activated around birth. As myelination proceeds, Krox-24 expression decreases in myelinating SC, while it is maintained in non-myelinating SC. In contrast, Krox-20 expression marks myelinating SC until adulthood (Topilko *et al.*, 1997). This

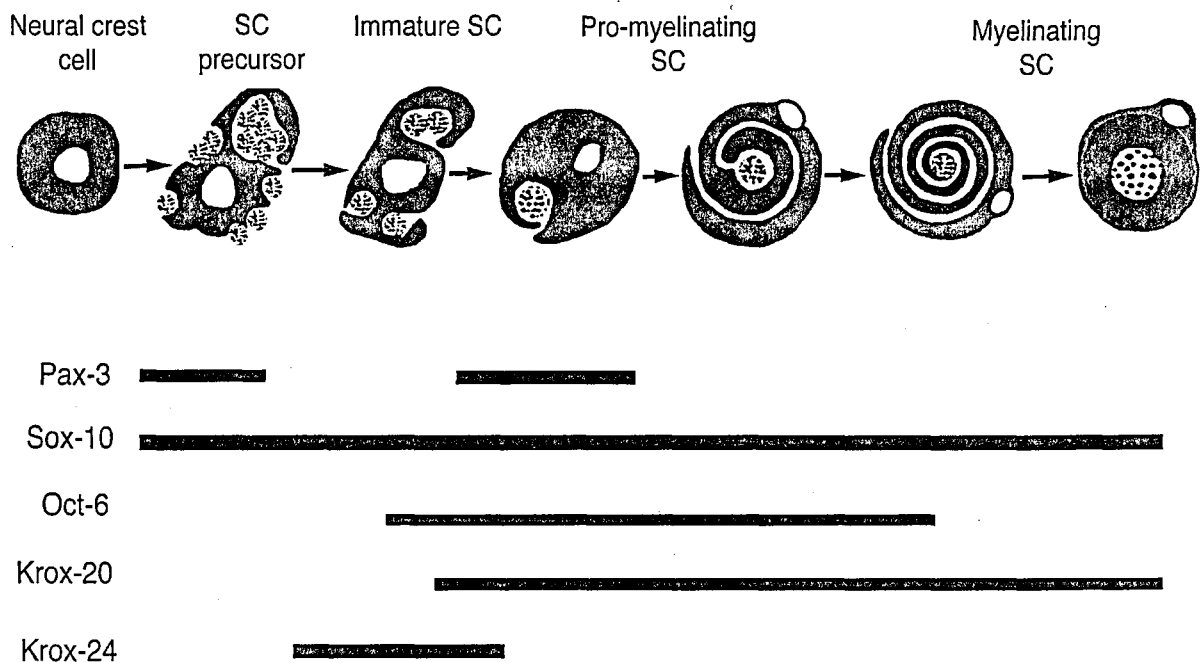


differential regulation is evident also after nerve damage. During Wallerian degeneration SC lose their myelin sheath and they de-differentiate to an immature SC phenotype, concomitantly *Krox-24* is upregulated, while *Krox-20* is turned off. Nerve crush experiments demonstrated that both genes are axonally regulated (Topilko *et al.*, 1997). This was shown also by in vitro study in which *Krox-20* expression is lost when SC are grown without axons, but its expression is restored in a co-culture system with sensory DRG neurites. The signal that allows *Krox-20* induction is a diffusible factor, in fact it can be restored by growing pure SC in the presence of a conditioned medium with Neuregulin, or in a combination with CNTF or bFGF (Murphy *et al.*, 1996).

The ablation of the *Krox-24* gene does not reveal any phenotype in SC (Lee *et al.*, 1996). On the contrary mice with a targeted deletion of *Krox-20* die soon after birth, and in addition to defects in hindbrain segmentation and in bone formation, they are defective in SC differentiation (Topilko *et al.*, 1994; Schneider-Maunoury *et al.*, 1993). All SC in these mice acquire a one-to-one relationship with the axon, but they seem to be arrested at the pro-myelinating stage. This impairment is also showed by a strong reduction in the expression of P0, MBP and PMP22 myelin genes. In the peripheral nerves of the few animals that survive after birth, both SC survival and apoptosis are increased as compared to control littermates. It is possible that *Krox-20* is involved in regulating cell-cycle exit into the pro-myelinating phenotype (Topilko *et al.*, 1994). The block at this stage causes an increase in SC, and subsequently in a competition for survival.

In humans, mutations in *Krox-20* are associated with peripheral neuropathies such as CMT, CH and DSS neuropathies (Warner *et al.*, 1998; Warner *et al.*, 1999). Co-transfection experiments show that *Krox-20* can partially trans-activate the P0 promoter (Zorick *et al.*, 1999). A recent paper describes an induction of mRNAs for P0, MBP, MAG, PMP22 Cx32 and MAG by *Krox-20* (Nagarajan *et al.*, 2001). They showed that some of the mutations described in human have a dominant negative effect on the wild type *Krox-20*, affecting, at the end, the expression of myelin genes. Because until now there is no evidence of a functional binding of *Krox-20* to any of myelin gene promoters, the modulation of the wild type activity by these mutants is probably associated with their ability to sequester *Krox-20* co-activators or co-repressors that are crucial for its function. Moreover, at the level of protein, these

Krox-20 mutants are more stable, strongly suggesting that this enhanced stability causes an increased expression that is sufficient to interfere with wild type Krox-20. Another mutation that causes human neuropathy is recessive and is located in the Krox-20 domain interacting with the NAB transcriptional co-factors (Warner *et al.*, 1999). The location of this mutation strongly suggests that NAB proteins are required for a correct modulation of Krox-20 on myelin genes. The recent finding that NAB can also be activators and not only repressors, depending on the specific promoter context, suggest that they may be responsible for the different effects of mutant Krox-20 proteins on individual promoters. Moreover both NAB1 and NAB2 contain multiple Egr binding sites, suggesting also a feed back regulation mechanism. (Sevetson *et al.*, 2000; Mechta-Grigoriou *et al.*, 2000).



**Figure 1.9**

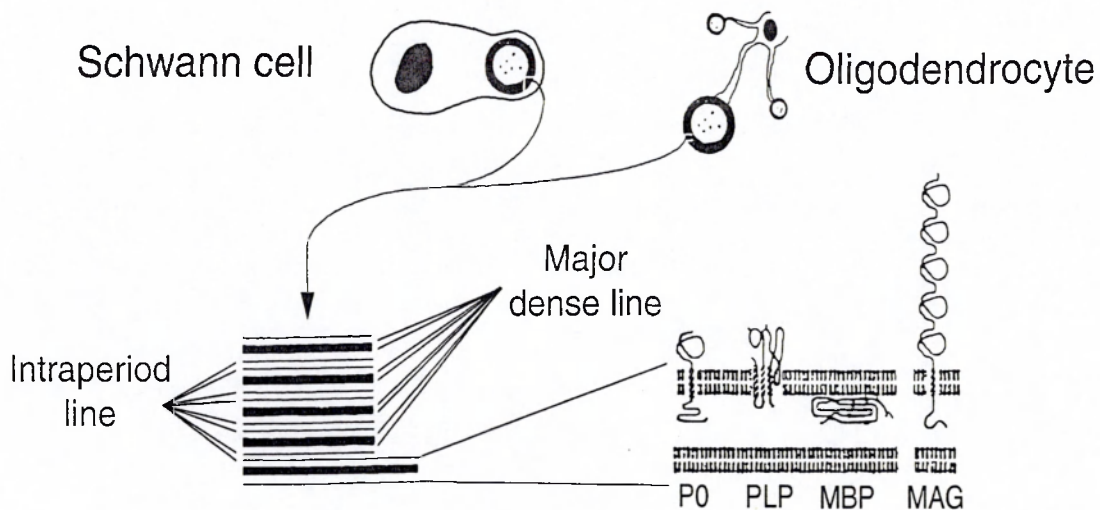
Schematic representation of the expression profile of transcription factors involved in Schwann cell development (Modified from Topilko P. and Meijer D., Glial cell development 2<sup>nd</sup> Edition Oxford University Press, 2001)

## 1.6 MYELIN

Myelin, one of the fundamental adaptations of vertebrates, is a multilamellar structure deriving from the spiral wrapping around an axon which in PNS has to be larger than  $1\mu\text{m}$  in diameter (Scherer, 1997; Martenson, 1992). Biochemical analysis revealed that myelin membrane is enriched in lipids composition; in fact it contains 70% lipid and 30% protein, with a high concentration of cholesterol and phospholipid; whereas usually plasma membranes contain 50% of lipids and 50% of proteins. CNS and PNS myelin include specific and overlapping sets of proteins (Martenson, 1992). They both contain high amounts of sphingolipids, including galactocerebroside and sulfatide. In compact myelin the membranes adhere to one another at both the cytoplasmic surfaces and the extracellular surface, giving rise respectively to the major dense line and the intraperiod line (Fig. 1.10). The alternating intraperiod and major dense lines give myelin its characteristic periodicity which is approximately 14nm in conventional preparation for electron microscopy while in fresh nerves prepared for X-ray diffraction it is approximately 18nm. The adhesion is stabilised by a combination of non-specific forces such as van der Waals attraction and electrostatic repulsion and specific short-range interactions mediated by protein. The major dense line can be interrupted by Schmidt Lanterman incisures, which appear in longitudinal section in a V-shaped configuration. These structures are much more visible in PNS than in CNS. They constitute a continuous channel of cytoplasm, which extends through the sheath from the periaxonal process to the soma. The incisure membrane is characterised by the presence of tight junctions, adherens junctions and gap junctions. Tight junctions separate paranodes and incisures from extracellular space. Adherens junctions link together consecutive layers of the myelin sheath. Gap junctions mediate a pathway of diffusion across incisures by forming a radial pathway for ions and small molecules (Balice-Gordon *et al.*, 1998). In contrast, CNS myelin has a radial component which is a network of interlamellar junctions running parallel to the nerve fiber axis and radially through the stacked membrane. It is essentially composed of tight junction proteins, in particular by claudin-11/oligodendrocyte-specific protein. Myelinated axons are completely covered by myelin sheaths except at nodes of Ranvier. Those are small gaps (less than  $1\mu\text{m}$  in length), directly exposed to the extracellular components.

The molecular components of CNS myelin partially overlap that of the PNS. The organisation of the axon itself is quite similar in the CNS and in the PNS (Fig. 1.10). However while SCs make a one to one relationship with the axons before myelinating, each OL can take contact with many axons whose number varies from tract to tract and appears to be related to axonal caliber. OL make fewer sheaths in tracts containing large myelinated fibers. They do not have a basal lamina or microvilli, like SC. OL express two proteins that are not present in SC, the Myelin Oligodendrocyte Protein (MOG) on their outer cell membrane (Brunner *et al.*, 1989), and Myelin Oligodendrocyte Basic Protein (MOBP) in the major dense line (Holz *et al.*, 1996). The principal myelin proteins expressed in the PNS are Protein Zero (P0), Peripheral Myelin Protein 22KD (PMP22) and MBP (Fig. 1.10).

Given the importance of myelin proteins in regulating the final structure of myelin I will discuss them in much more detail in a separate section.



**Figure 1.10**

Schematic diagram of the myelin structure in the CNS and in the PNS. The major myelin proteins involved in its formation and maintenance are indicated. See text for details.

## 1.7 MYELIN PROTEINS

In this section I will introduce not all the proteins that are constituents of myelin, but only those in which some of components of the transcriptional regulator mechanisms were identified.

### *MYELIN PROTEIN ZERO (P0)*

P0 is the major PNS myelin protein, comprising approximately 50% of all PNS protein (Greenfield *et al.*, 1973; Lemke *et al.*, 1988). It is the main adhesive molecule in compact myelin and it has a single extracellular IgG-like domain that it is thought to hold together the extra-cellular surfaces of compact myelin. P0 mRNA can be detected in a population of migrating neural crest cells, as well as in SC starting from E14, and in embryonic SCs prior to myelination (Lee *et al.*, 1997). Post-natally it is strongly upregulated in myelinating SC, both at the level of message and protein. The presence of P0 in early stages of development, suggests that it can play a role not only in the myelination process, but that it can be involved also in other mechanisms, such as cell-cell interactions in early glial cell development although *P0*<sup>-/-</sup> mice show no pre-natal phenotype. The X-ray resolution of the P0 protein structure suggests that in myelin, it forms tetramers in the plane of the membrane and that these tetramers can interact with their counterparts on the opposing cell membrane (Shapiro *et al.*, 1996).

In humans, mutations in the *P0* gene cause different demyelinating neuropathies: CMT-1B, CH and DSS. The mechanism through which these mutations act is different. Many observations, particularly in transgenic mice, suggest that the majority of these mutations operate at least in part through a gain of function mechanism. In some cases the mutant P0 is translocated to the membrane where it can interfere with the normal P0 protein and completely alter its function. In other cases, the mutation may act through a loss of function mechanism (Previtali *et al.*, 2000; Warner *et al.*, 1995).

SC in *P0* null mice can still form a multilamellar spiral around axons, but myelin is uncompacted, producing reduced nerve conduction velocity. The heterozygous *P0* null mice develop a late onset demyelinating neuropathy (Martini *et al.*, 1995). On the contrary *P0* overexpression causes a much more severe phenotype in

mice, indicating that the amount of this protein in SC must be strictly regulated (Wrabetz *et al.*, 2000).

#### *PERIPHERAL MYELIN PROTEIN 22KDa (PMP22)*

PMP22 represents approximately 2-5% of total peripheral myelin. It is expressed in low quantities in several other organs, including neurons in CNS, especially during development (Parmantier *et al.*, 1997). Its expression is regulated by two different promoters (Suter *et al.*, 1994). PMP22 was recently shown to interact with P0 protein (D'Urso *et al.*, 1999) and recruited to intercellular plasma membranes. Both overexpression and deletions of the *PMP22* gene cause myelin defects, strongly supporting the idea that even the amount of PMP22 is strictly regulated. In humans, duplication of the genomic region, in which the *PMP22* gene is located, causes CMT-1A disease, while transgenic mice carrying 15-30 copies of the gene, completely lack peripheral myelin and their SC show impaired differentiation and marked proliferation (Magyar *et al.*, 1996). Heterozygous deletion of the *PMP22* gene in mice and human or a frame shift mutation in human cause a mild peripheral neuropathy, the Hereditary Neuropathy with liability to Pressure Palsy (HNPP). On the contrary mice completely lacking *PMP22* have a similar but more pronounced phenotype with both hypomyelination and hypermyelination in young animals and severe demyelination in older animals (Adlkofer *et al.*, 1995).

#### *MYELIN ASSOCIATED GLYCOPROTEIN (MAG)*

MAG is a minor myelin constituent, comprising approximately 1% and 0.1% of total myelin proteins in CNS and in PNS respectively. It is a glycoprotein of the Ig-superfamily with significant homology to N-CAM. An alternative splicing of the primary transcript gives rise to two MAG isoforms, S-MAG and L-MAG which differ in their cytoplasmic domains (Fujita *et al.*, 1998). In rodents, MAG expression peaks early during development and declines thereafter both in CNS and in PNS. MAG interacts with a component of the extracellular matrix, tenascin R and with different types of collagen influencing the formation of collagen fibrils (Bachmann *et al.*, 1995; Probstmeier *et al.*, 1992). MAG immunoreactivity is detectable on OL processes before formation of compact myelin (Trapp *et al.*, 1989). On myelinating SC, MAG is

detected only after SC processes have turned at least once around the axon (Martini and Schachner, 1986). After myelin formation MAG is restricted to the periaxonal, paranodal and Schmidt-Lanterman regions (Arroyo and Scherer, 2000). Based on cell culture experiments, it has been suggested that MAG can mediate the interactions between myelinating SC and neurons and it can play a role in the initiation of myelination (Poltorak *et al.*, 1987; Sadoul *et al.*, 1990). Experimentally reduced levels of MAG in SC-DRG co-culture experiments renders SC unable to segregate large caliber neurites and impaired myelination (Owens and Bunge, 1991). Conversely experimentally increased levels of MAG in SC accelerated initial ensheathment of DRG neurites (Owens *et al.*, 1990). However *MAG* null mice showed a delay in the formation of compact myelin only in optic nerves of young animals while in PNS the initiation of myelination was not affected (Montag *et al.*, 1994). The analysis of adult and aged *MAG*  $-/-$  mice revealed degenerative alterations of myelin in the PNS and to a minor extent in the CNS, with the formation of classical onion bulbs in the peripheral myelin. Those alterations support an *in vivo* role for MAG in the long-term maintenance of myelin integrity, at least in PNS.

#### *PROTEOLIPID PROTEIN (PLP/DM20)*

The PLP/DM20 protein constitutes approximately 50% of the total CNS myelin protein, and it is a minor component also of the PNS myelin (Garbern *et al.*, 1997). The gene gives rise to two alternatively spliced transcripts that encode the classical PLP gene and the smaller isoform DM20. The only difference between them is the presence of an additional 35 aminoacids in the intracellular region of PLP. The *plp/dm-20* gene is expressed primarily by mature OL, although small amounts of the protein products have been detected in embryonic CNS, PNS, heart, spleen, thymus and lymphnodes (Martenson, 1992; Bongarzone *et al.*, 1999; Bronstein, 2000a). *PLP* duplication is a relatively common cause of Pelizaeus-Merzbacher disease in man and results in a fairly mild phenotype, although larger duplication of the genomic region can result in more severe clinical manifestations (Inoue *et al.*, 1999). In mice, 2-fold overexpression causes severe hypomyelination, astrogliosis, seizures, and premature death (Readhead *et al.*, 1994). OL in these animals contain vacuoles with a variety of myelin proteins, suggesting that dysmyelination is a result of protein missorting and subsequent inability

to assemble myelin (Anderson *et al.*, 1999). Surprisingly disruption of the gene as well as spontaneous deletion or mutations, with a complete lack of the protein, result in relatively mild disease. PLP/DM20 null mice have normal compact myelin but the ultrastructural analysis revealed condensed intraperiod lines and reduced stability (Klugmann *et al.*, 1997; Boison and Stoffel, 1994). There are two spontaneous mutant mice, *jimpy* and *msd*, in which point mutations cause premature OL death and subsequent inability to form normal myelin. OL of these mutants have perinuclear inclusions, which probably represent an accumulation of mutant protein (Gow *et al.*, 1998). Recently transgenic mice expressing only the DM20 isoform were created. These mice have altered CNS myelin periodicity and older animals develop abnormalities in coordination, although the DM20 isoform was normally targeted in myelin, suggesting that the aminoacids not present in DM20, are not essential for myelin structure and function (Stecca *et al.*, 2000).

#### *OLIGODENDROCYTE-SPECIFIC PROTEIN (OSP/CLAUDIN-11)*

In CNS myelin there are two OSP/claudin-11 transcripts of approximately 2Kb and 4Kb and they probably derive from a single gene. In adult, these proteins contribute 7% of total CNS protein and they are also present in Sertoli cells. Embryonically OSP is widely expressed in the mesenchymal cells adjacent to chondrocytes, in developing meningeal cells and in OL precursor cells before myelination starts (Bronstein *et al.*, 2000b; Morita *et al.*, 1999). Low level expression was also found in the human PNS. The *osp/claudin-11* gene contains three exons (Bronstein *et al.*, 1996). The ablation of *osp/claudin-11* gene in mice abolishes tight junctions in CNS and in Sertoli cells, leading to neurological deficits and abnormal formation of the testes. However myelin was grossly intact even if animals show reduced nerve conduction velocity and difficulties in coordination (Gow *et al.*, 1999).

#### *MYELIN-OLIGODENDROCYTE GLYCOPROTEIN (MOG)*

MOG is a myelin glycoprotein preferentially expressed at the outer surface of the myelin sheath and OL processes, with only low expression in the compact myelin (Johns and Bernard, 1999). It is a quantitatively minor component of myelin representing 0.05-0.1% of all myelin proteins. Immunocytochemical studies on



cultured OL showed that MOG is expressed 24-48 hours after other myelin proteins. MOG shows a caudo-rostral gradient of expression that is typical of all myelin genes both at the mRNA and at the protein level. Based on its secondary structure MOG can be considered an adhesion molecule even if there is no direct evidence supporting this function. It was speculated that MOG might have an immune function in the CNS. Many studies were performed to correlate this hypothesis with the generation of diseases like multiple sclerosis, suggesting that MOG does not have a direct role in the myelination processes, rather it mediates interactions with the immune system.

## 1.8 TRANSCRIPTIONAL CONTROL OF MYELIN GENES

In the case of the genes encoding for the myelin proteins, very little is known of their transcriptional regulation. Many studies have tried to identify transcription factors that are relevant in controlling the expression of myelin genes, because they are predominantly regulated at the transcriptional level. Classical transcription studies, aimed at identifying cis-acting sequences present in the promoter and transcription factors able to bind them, were performed on the principal myelin proteins expressed both in CNS and in PNS and they led to the identification of some transcription factors.

### *PLP*

One of the first transcription factors known to be involved in the regulation of a myelin gene, was identified in the proximal promoter of the *plp* gene. This factor is a zinc finger transcription factor named MyT1 and it recognises the region from -242nt to -232nt of the proximal PLP promoter (Kim and Hudson, 1992). MyT1 is expressed in OPC both in vivo and in vitro. The other factor that was recently shown to be involved in the regulation of the PLP promoter is Gtx. It is an homeodomain transcription factor that binds also in 750nt of the proximal MBP promoter. In the PLP promoter Gtx can bind four cis DNA elements, located in 1.3Kb of the promoter (Awatramani *et al.*, 1997). The precise role of these transcription factors in regulating the PLP gene is still controversial. In fact the expression of MyT1 declines immediately after the cells differentiate and begin to myelinate, and Gtx is expressed only in completely differentiated OL. Nevertheless a recent paper suggest that Gtx can be a repressor in glial cells (Awatramani *et al.*, 2000).

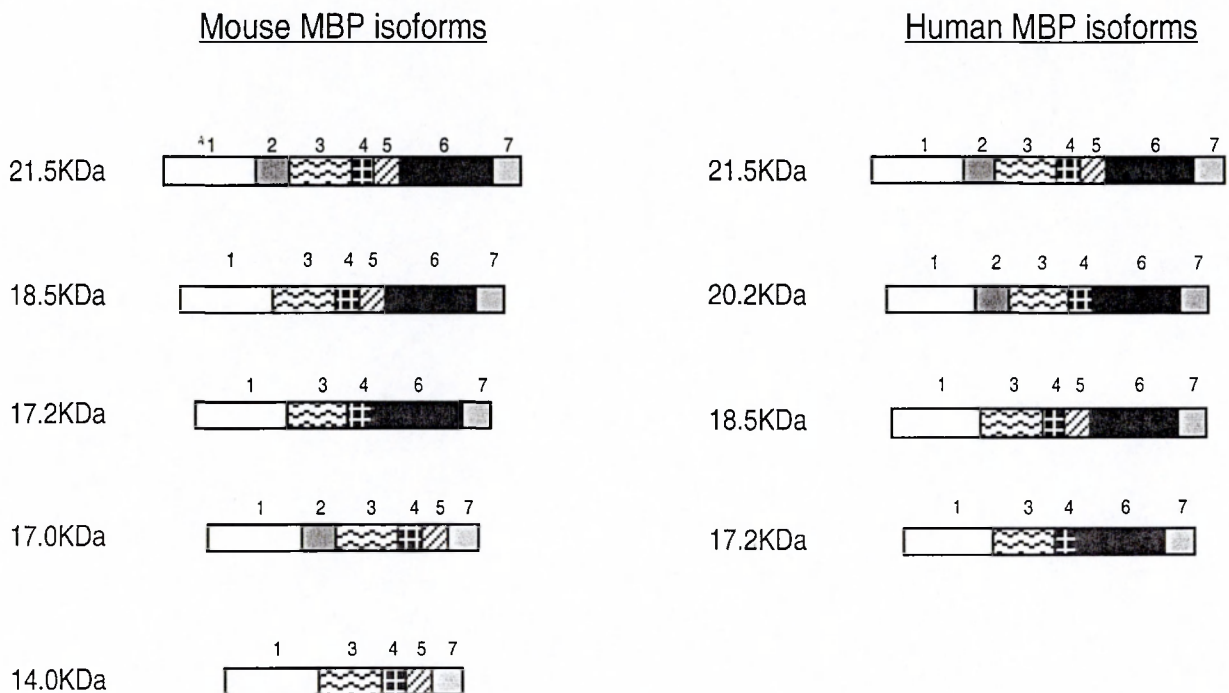
### *P0*

The analysis of the proximal 1.1Kb of the P0 promoter led to the identification of strong conservation between human, mouse and rat, of 350 nucleotides proximal to the transcription start site. DnaseI footprinting and EMSA analysis on the promoter revealed the presence of binding sites for the transcription factors NF-Y and Sp-1 (Brown and Lemke, 1997). Another transcription factor that has an effect on the in vitro activation of the same region of the P0 promoter is Oct-6/SCIP/Tst1. In vitro

studies showed that it could be considered as a repressor not only for P0 but also for MBP (Monuki *et al.*, 1993). Subsequent *in vivo* studies are not consistent with a repressive role of Oct-6 on myelin genes, but as we previously discussed, it is possible that Oct-6 has two separate functions. Recently, another transcription factor known to be involved in SC development, Sox-10, was shown to transactivate the 1.1Kb fragment of the P0 promoter. Unfortunately all the analyses were performed in the N2A cell line, which do not express myelin genes, and not in SC. Nevertheless the authors showed that Sox-10 dependent induction is specific for P0 (Peirano *et al.*, 2000).

## 1.9 THE MBP GENE

MBP comprises approximately 35% of total protein in CNS myelin and 15% in PNS myelin. It has been found in all vertebrates that have myelin, both in the CNS and in the PNS. It is localised in the major dense line of myelin and in CNS it is required for normal myelin compaction. The analysis of a natural mouse mutant, the shiverer (*sh*) mouse, shows that an intragenic deletion of the 3' region of the gene causes severe hypomyelination, a delamination in the CNS major dense line, tremors and tonic seizures (Molineaux *et al.*, 1986). Although MBP is also expressed in the PNS, the peripheral myelin in these animals is relatively normal, suggesting the presence of another myelin gene in PNS that can rescue its absence. The *mbp* gene is composed of seven exons that span a region of 32Kb in the mouse genome. When the mRNA retains all the exons the primary transcript gives rise to a protein of 21.5KDa. Alternative splicing of the exons produces various MBP isoforms (Fig. 1.11). In mouse, for example, there are other isoforms of 18.5KDa (exon 2 excised), 17KDa (exon 6 excised) 17.2KDa (exons 2 and 5 excised) and 14KDa (exons 2 and 6 excised). The 18.5KDa and the 14KDa isoforms are the most abundant proteins present in the adult mouse CNS (Barbarese *et al.*, 1978). The human *mbp* gene is composed of seven exons spanning a region of 40-45Kb. Its analysis revealed the existence of four isoforms, the 21.5KDa, with all seven exons, the 20.2KDa (exon 5 excised), the 18.5KDa isoform (exon 2 excised) and the 17.2KDa isoform (exons 2 and 5 excised) (Kamholz *et al.*, 1988) (Fig. 1.11). The molecular basis for this pattern of alternative splicing is not known. Comparison of the splice junction sequences surrounding the exons showed that there are no significant differences between them. Human and mouse primary transcripts were shown to be structurally similar, since they are both transcribed from a unique site in the genome within a single promoter and are polyadenylated at similar positions. The accumulation rates of these protein isoforms change during development and it is due to a modulation of their mRNA levels (Carson *et al.*, 1983). Between day 10 and day 30, the most active period of myelination, the molar ratio among the 21.5KDa:18.5KDa:17KDa: 14KDa isoforms is 1:5:2:10. In later phases of mice life the ratio between these isoforms changes to 1:10:3.5:3.5 (Barbarese *et al.*, 1978).



**Figure 1.11**

Schematic diagram of the MBP isoforms present in the mouse and human gene. The formation of each isoform is due to alternative splicing of MBP exons. The exons maintained in each isoform are indicated. These isoforms are differentially expressed during development. See text for details.

The classical *mbp* gene is a part of a much larger gene, named *golli-mbp*, which is composed of 11 exons. *Golli-mbp* is expressed in brain regions rich in oligodendrocytes and their precursors, in neurons and also in organs of the immune system. Interestingly not only MBP exons but also the classic MBP promoter overlap the coding region of some *Golli-mbp* transcripts (Campagnoni *et al.*, 1993).

Nuclear transcription run on assays showed that the developmental expression of MBP is primarily regulated at the level of transcription (Wiktorowicz and Roach, 1991). The total amount of MBP mRNA increase during the myelination process. The levels of these mRNAs increases 10- to 100-fold between day 2 and day 30 after birth, and fall to approximately 40% of this peak in adult (Wiktorowicz and Roach, 1991). Moreover the steady state level of MBP mRNA increases in parallel with protein, and both precede the appearance of compact myelin (Lemke and Chao, 1988; Zeller *et al.*, 1984). Unlike SC, OL are able to initiate MBP transcription in vitro in the absence of axons, although in vivo, in transgenic mice, the proximal MBP promoter is significantly axonally regulated (Wrabetz *et al.*, 1998). Although the myelin proteins appear at different times during OL development, the mRNAs encoding these proteins accumulate with similar temporal profiles both in the developing brain and in OL in culture (Zeller *et al.*, 1984; Scherer *et al.*, 1994a). In brain they are detected at birth in brainstem of rodent animals, and subsequently, appear in the forebrain, following a caudo-rostral gradient.

Analysis of MBP transcriptional regulation has been studied both in vitro and in vivo, in transgenic mice. Transgenic mice were generated in order to define the minimal region at the 5' of the gene that confers the precise tissue specificity and developmentally regulated expression of a heterologous gene. In transgenic mice this strict regulation can be achieved with as small a portion as 256bp of the MBP promoter, but only in OL and not in SC. All transgenic mice generated up to now, with different parts of the promoter from 9.0Kb to 256bp, activate regulated MBP or heterologous cDNA expression in the CNS (Forghani *et al.*, 2001; Foran and Peterson, 1992; Goujet-Zalc *et al.*, 1993; Goverman *et al.*, 1993; Gow *et al.*, 1992; Hayes *et al.*, 1992; Jensen *et al.*, 1993; Katsuki *et al.*, 1988; Kimura *et al.*, 1989; Miskimins *et al.*, 1992; Readhead *et al.*, 1987; Zingernakel *et al.*, 1990; Wrabetz *et al.*, 1998). However the analyses of more distal regions of the MBP promoter revealed the existence of a specific PNS

enhancer located between -9.0Kb and -8.4Kb and named Schwann Cell Enhancer 1 (SCE1), that activates transcription only in SC (Forghani *et al.*, 2001). This is the first region that was shown to be both necessary and sufficient to obtain a correct pattern of expression of MBP in SC. The same researchers found a more distal region located between -12.0kb and -9.0Kb of the MBP promoter region, named SCE2, that can direct the expression of the reporter gene in SC, even if the analysis of the transgenic mice was restricted only to one line. These *in vivo* studies confirm that the transcriptional machinery that regulates the expression in OL and in SC is different.

The possibility of obtaining MBP expression in OL in cultures, in the absence of axons (Zeller *et al.*, 1985), allowed the *in vitro* analysis of the MBP promoter in this glial cell type. This analysis was restricted to the identification of cis-acting elements lying in the proximal region of the MBP promoter and the trans acting factors binding them. One approach used to identify important DNA elements was to compare these sequences between different species or, with that of other myelin genes. These studies allowed the identification of a strong conservation in the first 150bp upstream of MBP. The start site of transcription is located, both in mouse and in human, 48bp upstream from the translational start site. No TATA or CAT boxes are present in either promoter, although both contain a TATA-like sequence, TTCAAA, 30bp upstream of the cap site, and a CAT-like sequence, CCACTT 80bp from this site. Two other elements were found also in promoters of other myelin genes, a sequence located at -96bp, which is present also in the PLP and in the P0 promoters, and 12bp located at -53bp which are present also in the PLP promoter. Unfortunately the functional significance of these elements is not known.

The analysis of the first 149bp of the human MBP promoter in primary culture of OL allowed the identification of two functional regions. A positive region is located proximal to nucleotide -102 and it is active both in cell types that do not transcribe MBP and in primary developing OL cultures (Wrabetz *et al.*, 1993). Another region from -149bp to -102nt is a repressive element in Cos7 cells, while in primary OL there is a negative element between -116nt to -124nt and a positive element from -124nt to -149nt (Taveggia *et al.*, 1998). Interestingly Aoyama *et al.* showed that from -124nt to -110nt, there is a consensus binding site for a family of transcription Nuclear Factor 1 (NF-1) encoded, at least, by four genes differentially expressed in brain (Aoyama *et al.*,

1990; Chaudhry *et al.*, 1997). We also described a sequence upstream and partially overlapping the NF-1 binding site, which activates the MBP promoter in primary OL but not in Cos7 cells. A complex of protein enriched in brain, OL and SC, named MEBA, binds this sequence (Taveggia *et al.*, 1998). The analysis of the more proximal region of the MBP promoter allowed the identification of another region, named MB1, from -14nt to -50nt, which is bound by Pur $\alpha$ , a 39KDa protein. This is a sequence specific single stranded DNA binding protein that has a high affinity for the purine rich motif (Haas *et al.*, 1993; Haas *et al.*, 1995). Recently another transcription factor Gtx, belonging to the homeodomain family of transcription factors, was shown to bind inside 750nt of the proximal MBP promoter and in 1.3Kb of the PLP promoter region. Gtx is a sequence specific DNA binding protein that binds to DNA sequences containing a core AT rich homeodomain binding site. It is exclusively expressed in differentiated OL and not in SC or precursor OL and astrocytes and its mRNA increases in parallel with that of MBP and PLP (Awatramani *et al.*, 1997). Apart from the attempts performed to clarify the transcription mechanism of the MBP gene, the integrated functions of all of them are still obscure.



## Chapter 2

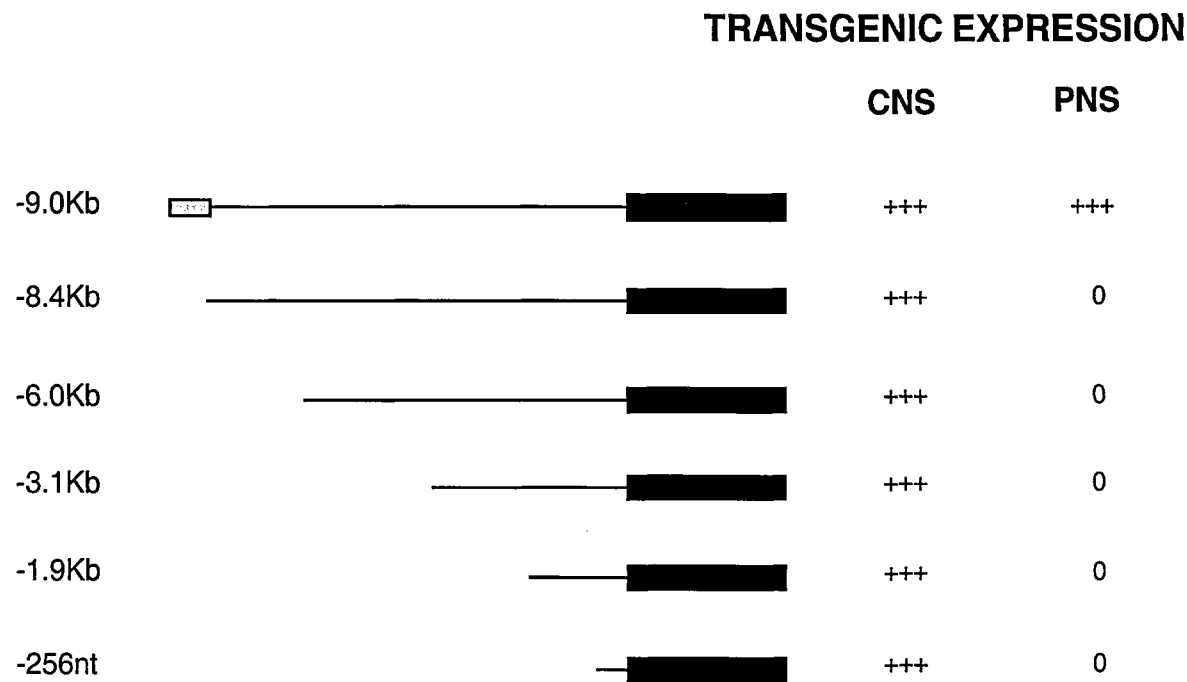
# RESULTS

### 2.1 IN VITRO ANALYSIS OF A SC MBP ENHANCER IN VITRO

#### *FUNCTIONAL ANALYSIS*

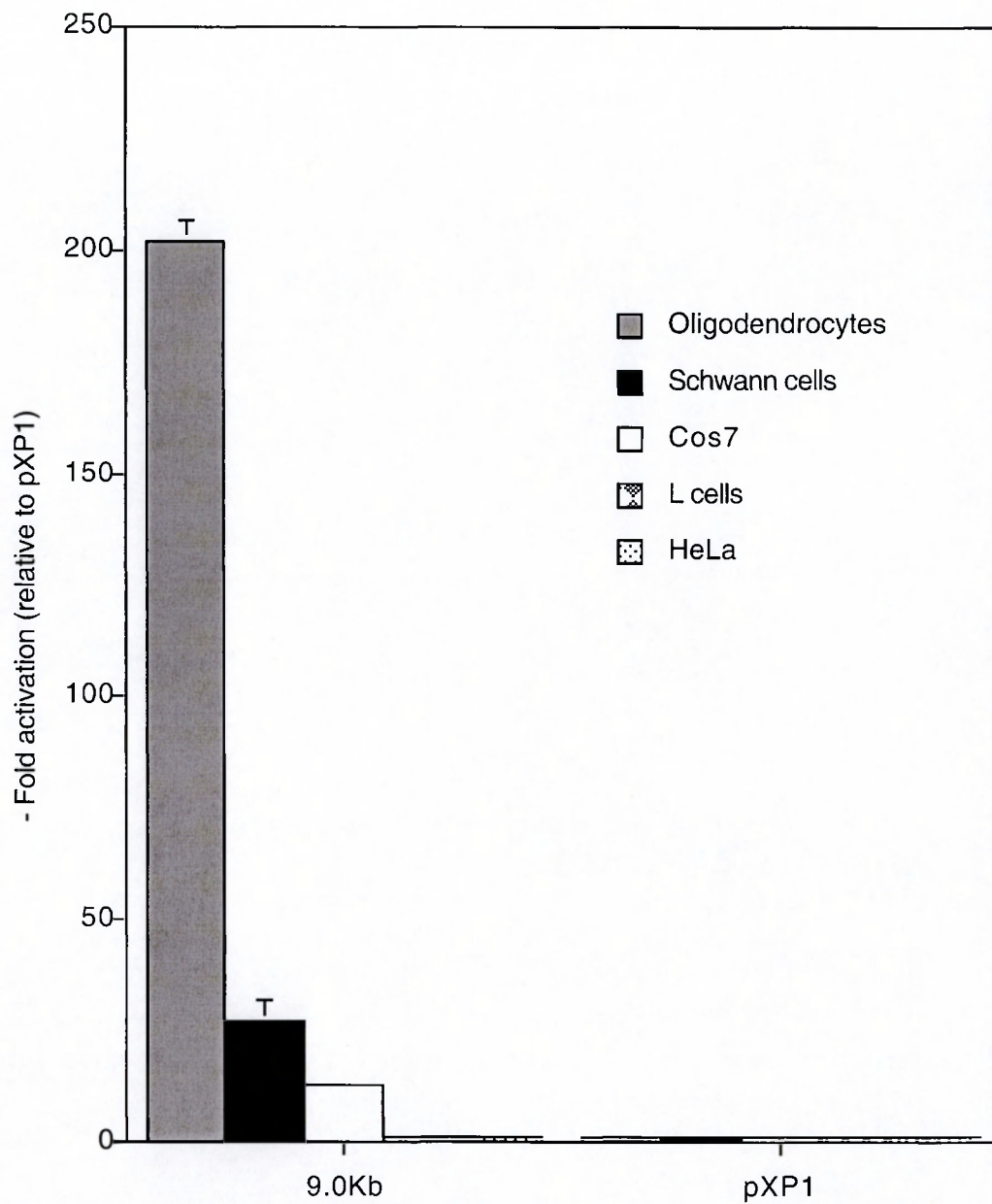
Previous analysis performed in transgenic mice and in cultured cells showed that transcription of MBP is regulated by different cis-acting elements and trans-acting factors in SC as compared to OL. In particular, A. Peterson and colleagues identified a 588nt region 9Kb upstream of the start of MBP transcription required to activate transcription in SC but not in OL. Whether this region contributes to MBP transcription in OL was not determined (Forghani *et al.*, 2001). Therefore we analysed the extended MBP regulatory region in cultured cells to address two questions: does the 588nt region activate transcription in both SC and OL; and can cultured SC provide the means to dissect cis-acting elements within the 588nt region.

We generated a series of reporter constructs containing the same MBP regulatory region analysed in transgenic mice: 9.0Kb, 8.4Kb, 6.0Kb, 3.2Kb, 1.9Kb and 256bp. Even if MBP is transcribed robustly both in CNS and in PNS, only transgenic mice containing the region from 9.0Kb express the *lacZ* reporter gene both in CNS and PNS. All other constructs activate the reporter gene only in CNS (Forghani *et al.*, 2001). We cloned all of these fragments upstream of the luciferase gene, used as a reporter gene, in the pXP1 promoterless plasmid, and we measured their activity after transient transfection of primary OL and SC. (Fig. 2.1)



**Figure 2.1**  
Schematic representation of the constructs previously used to generate transgenic mice, with their relative expression in Central and Peripheral Nervous System. The same constructs were used in our analyses. Numbers indicate MBP promoter regions inserted in the constructs. The dark grey box indicates the enhancer element located between 9.0 and 8.4Kb upstream of the transcription start site. Black boxes represent the reporter gene, which is *lacZ* gene in the case of transgenic mice and luciferase in our study.

To measure-cell specific activation of the 9.0Kb MBP construct, we transiently transfected primary cultures of OL and SC, as well as cells that normally do not express MBP, including undifferentiated murine fibroblast L cells, a human carcinoma cell line, HeLa, and monkey SV40 transformed renal cells, Cos7. As shown in Fig. 2.2, 9.0MBP luc was specifically activated in OL and in SC. In OL it activated approximately 200-fold above the promoterless control plasmid, whereas in SC the expression was increased 27-fold above pXP1. On the contrary, L cells and HeLa cells did not show any significant activation mediated by 9.0Kb of the MBP promoter. In Cos7 cells we found that this construct was transactivated approximately 13-fold above the promoterless construct, even though MBP is not expressed in this cell line. It is possible that this activation is an artifact due to cell transformation. These data show that strong cell-specific activation of the 9.0Kb promoter is maintained both in primary cell culture of OL and SC, allowing us to set up a system in which to dissect the MBP promoter.

**Figure 2.2**

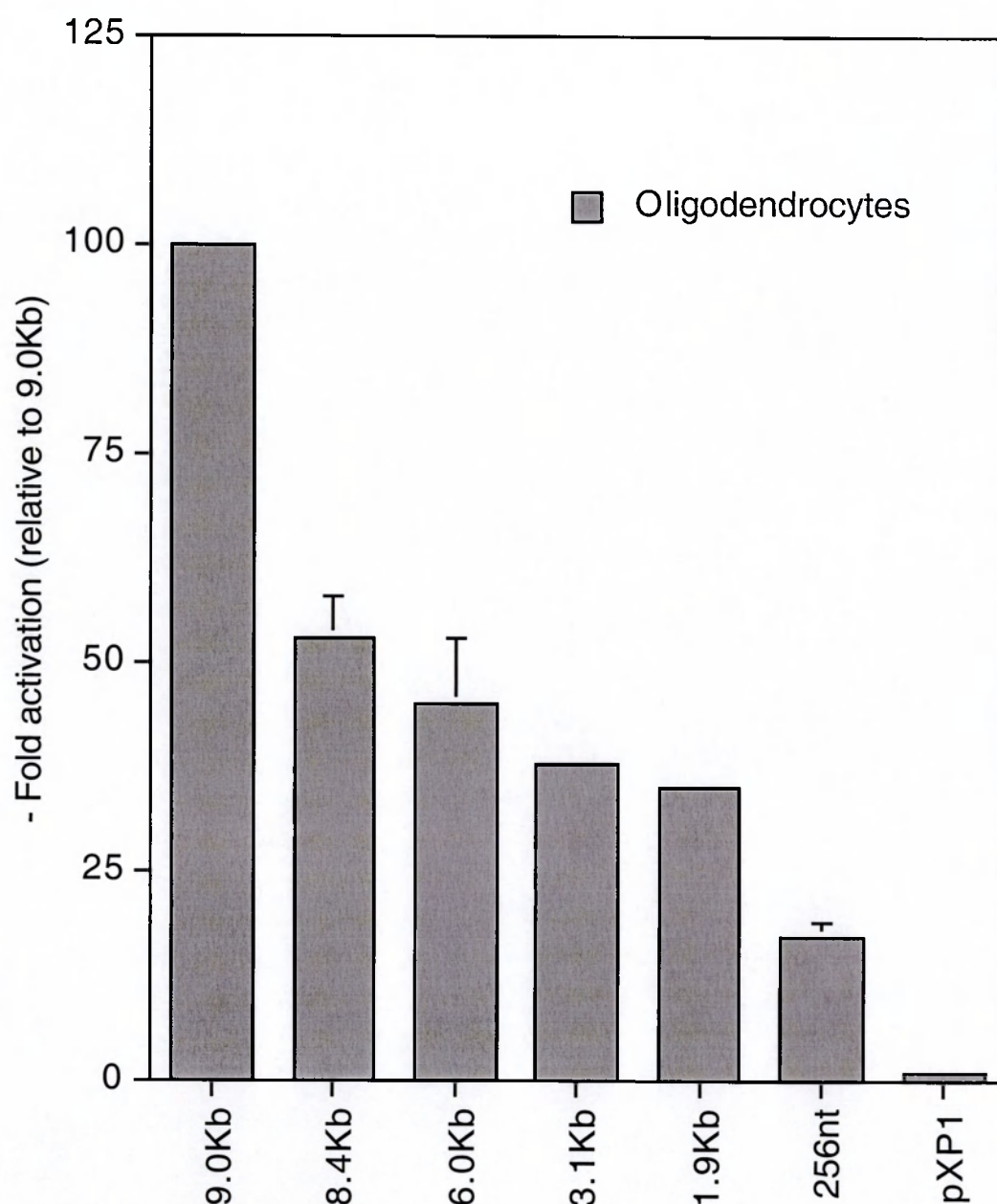
Transient transfection analysis of plasmids containing 9.0Kb of the MBP promoter in oligodendrocytes (OL), Schwann cells (SC), Cos7, HeLa and L cells. Fold-activation is expressed as a ratio between luciferase and  $\beta$ -gal activity as a measure of transfection efficiency. The final activity is relative to the promoterless construct pXP1 = 1.0. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

In order to identify putative regions important in regulating MBP expression within the 9.0Kb mouse promoter, we performed a DNase I hypersensitivity assay on nuclei prepared from brain. This is one of the principal methods used in mapping the accessibility of a genomic locus. The major advantage of this technique is that it allows the detection of regions that are bound by transcription factors *in vivo* and for this reason they can be relevant to direct transcription of a specific gene in a particular time of the development. Using this approach, a Schwann cell enhancer in the genomic locus of the Oct-6 gene was recently identified (Mandemakers *et al.*, 2000). We prepared nuclei from 30 day old mouse brains, and we treated them with increasing amounts of DNase I. We then extracted the DNA and performed Southern blot analysis. We digested DNA using BamHI, as previous genomic mapping showed that this restriction enzyme releases a 10Kb fragment of the MBP promoter with the start site of transcription at its 3' extremity (Takahashi *et al.*, 1985). The design of a probe inside intron 1 of the gene and at the 3' of the 10kb region allowed us to detect possible hypersensitive sites (HSS). Unfortunately, we were unable to distinguish any HSS despite multiple attempts. The proximal promoter should have served as a positive control, as we know that it is highly active in OL from brain at P28. This negative result can be due to the fact that in brain there are many cell types and OL represent only a small part of the total. We did not try the same experiments on nuclei prepared from sciatic nerve because of the difficulty of obtaining sufficient number of nuclei. Schwann cells in culture were also not examined because the level of MBP transcription required to detect HSS would likely require axonal contact.

To map regions important for activation within the 9.0Kb construct, we transiently transfected primary cultures of OL (Fig. 2.3a) and SC (Fig. 2.3b) with the same constructs previously used to generate transgenic mice. In OL, we found that deletion from 9.0Kb to 8.4Kb reduced the activity of the reporter gene approximately 50%. On the contrary the region from 8.4Kb to 1.9Kb had no significant effect. Deletion from 1.9Kb up to 256nt reduced the activity approximately 15%, and deletion of the proximal MBP promoter reduced the activity to background level. Thus, in agreement with previous results, we identified two regions important for the positive regulation of the 9.0Kb region: from 9.0Kb

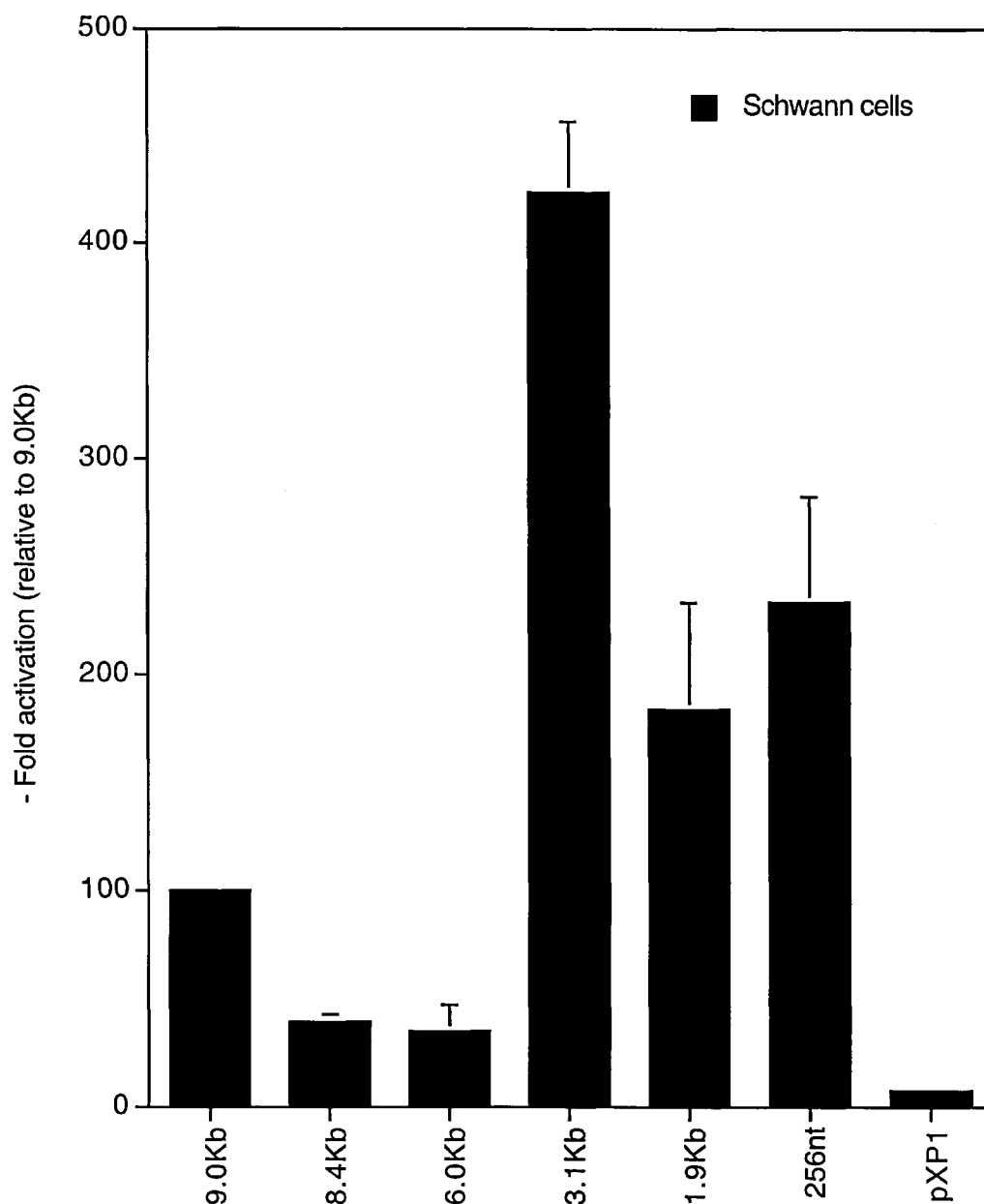
to 8.4Kb and the proximal part of the MBP promoter (Forghani *et al.*, 2001; Wrabetz *et al.*, 1993).

In SC, we found that deletion from 9.0Kb to 8.4Kb had a stronger effect than the one observed in OL; in fact there was a reduction of approximately 70%. Deletion from 8.4Kb to 6.0Kb had no significant effect. When we deleted to 3.1Kb we found an activation, which was 4-fold above the 9.0Kb region, and the same activation was maintained in deletion to 256nt. Finally, as in OL, removal of the proximal 256nt region reduced activity to background levels. The paradoxical activation of 3.1Kb, 1.9Kb and 256nt fragments was not observed in transgenic mice, as these constructs are not able to transactivate the reporter gene. This analysis strongly suggests that, among all these elements, two regions are important activators in 9.0Kb of the MBP promoter, both in OL and in SC. The first one is the proximal promoter and the other one is the region from 9.0Kb to 8.4Kb, which is an important activator in primary culture of both SC and OL.



**Figure 2.3a**

Transient transfection analysis of plasmids containing progressive deletions of the 9.0Kb MBP promoter in primary OL. The final activity is relative to 9.0Kb = 100. Deletion of the 588nt between 9.0Kb and 8.4Kb reduces the activity by approximately 50%, while deletion of the proximal MBP promoter region reduces the activity to background level. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

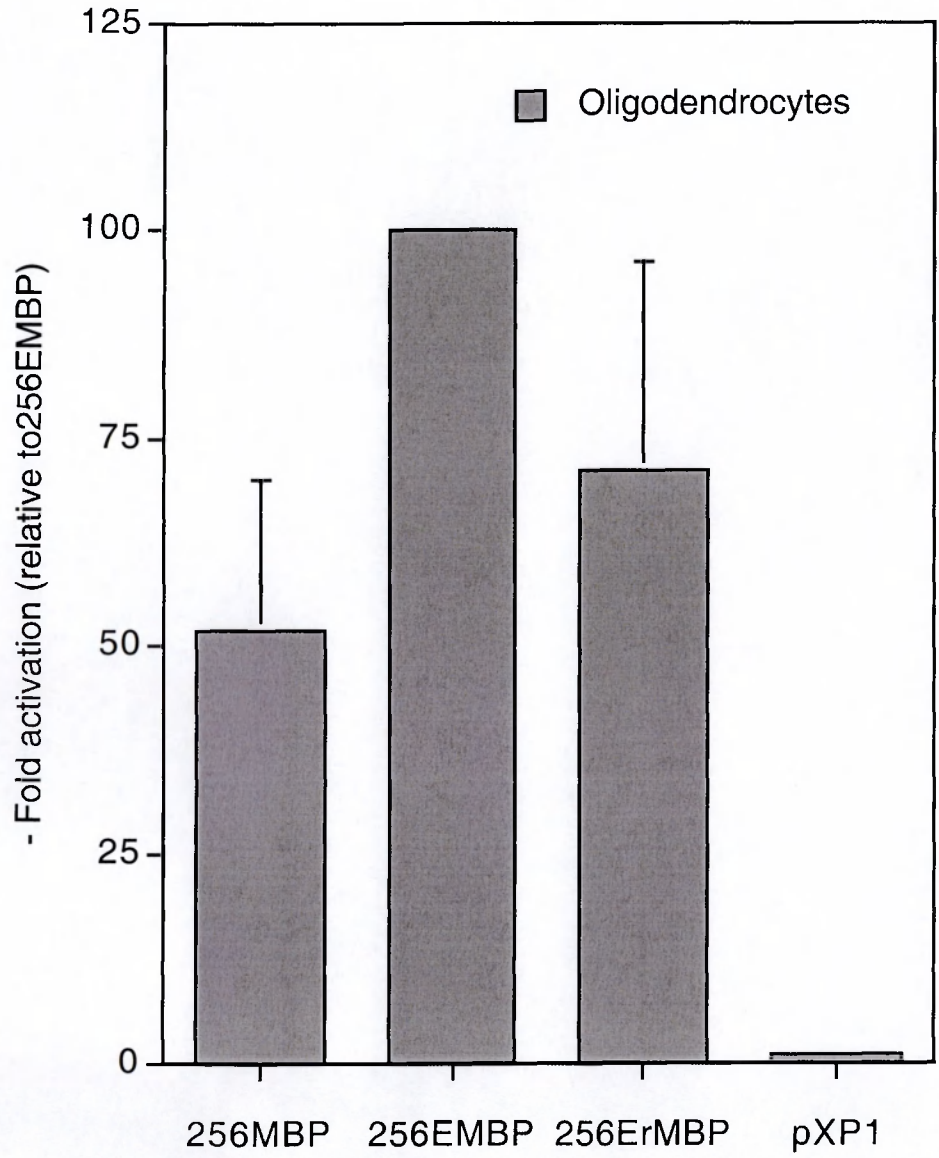


**Figure 2.3b**

Transient transfection analysis of plasmids containing progressive deletions of the 9.0Kb MBP promoter in primary SC. The final activity is relative to 9.0Kb = 100. Deletion of the 588nt between 9.0Kb and 8.4Kb reduces the activity by approximately 70%, while deletion of the proximal MBP promoter region reduces the activity to background level both in OL and SC. Of note, the 4-fold activation in the 3.1Kb construct was not present in transgenic mice. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

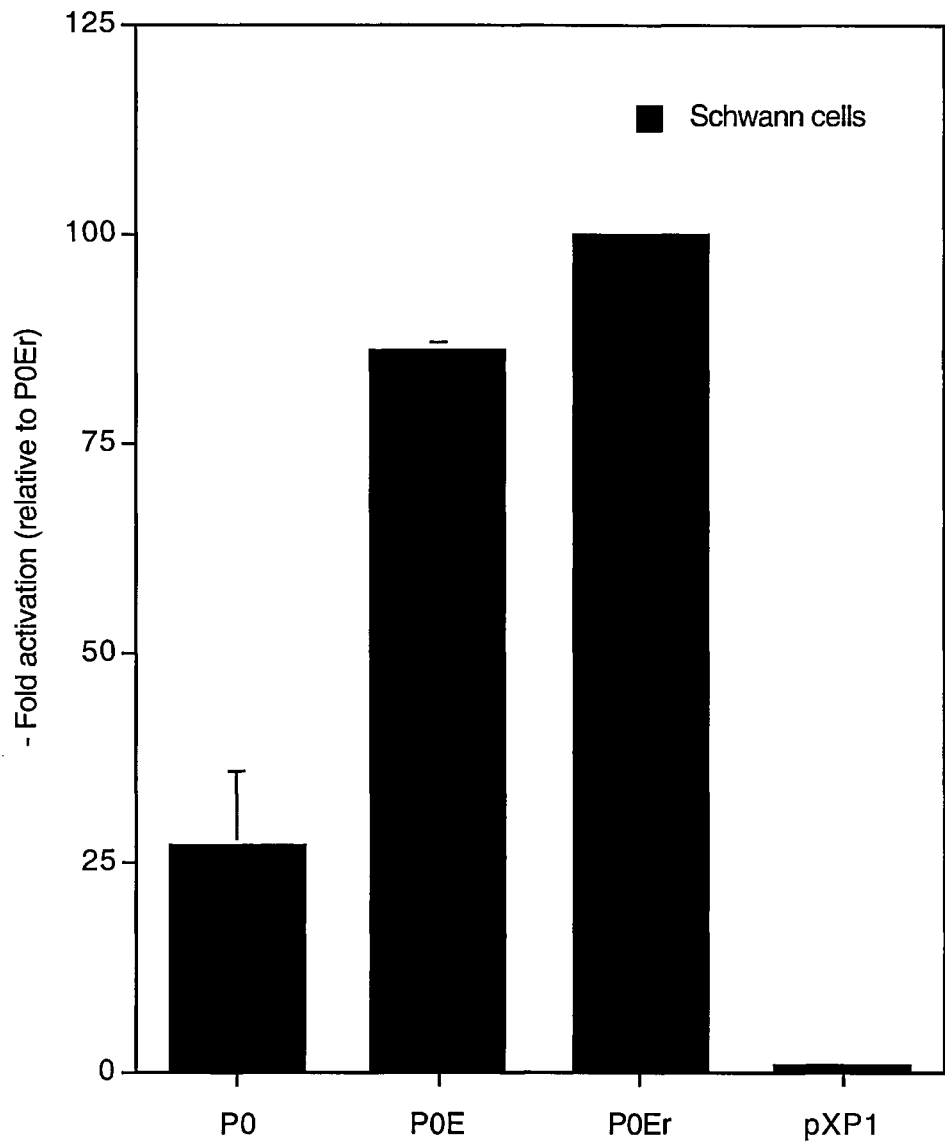


We determined if the 9.0-8.4 region was an enhancer in SC and OL. A classical enhancer activates a heterologous promoter in an orientation- and distance-independent fashion. For example, in the *in vivo* characterisation of 9.0Kb of MBP promoter, the 588bp lying between 9.0Kb and 8.4Kb, behaves as an enhancer only in SC. By cloning it, in both orientations, upstream of 300bp of the minimal hsp68 heterologous promoter, A. Peterson showed that even in transgenic this element is a SC enhancer, in fact the *lacZ* reporter gene is specifically activated only in SC and not in OL in spinal cord or brain. To validate our *in vitro* model, we cloned this region in both orientations, upstream of homologous and heterologous promoters, and performed transient transfection analyses of primary SC and OL (Fig. 2.4). As homologous promoter we chose the minimal MBP promoter (256nt). Because of the excessive activation found in SC, we were able to analyse these constructs only in OL (Fig. 2.4a). These transient transfection assays revealed that, in OL, constructs containing these 588nt are not able to significantly enhance the activation of the reporter gene (Fig. 2.4a). To test the enhancer activity in primary SC, we cloned the same region, upstream of another myelin specific promoter. We used the 1.1Kb region of the proximal P0 promoter that was previously shown to be specifically expressed in SC (Lemke and Chao, 1988; Brown *et al.*, 1997). These 588nt can upregulate the heterologous P0 promoter 3-4 fold in both orientations (Fig. 2.4b), strongly suggesting that it behaves as an enhancer in SC. Finally we also cloned this element upstream of the Thymidine Kinase promoter (TK109). This is a heterologous, non-myelin promoter that can be activated in both OL and SC. Constructs containing the 588nt, in either orientation, were transiently transfected in both cell types. In SC, the region activated the TK promoter approximately 3-fold above the construct containing only TK109, whereas in OL we never detected a transactivation above TK109 (Fig. 2.4c). These results strongly suggest that the region from 9.0Kb to 8.4Kb is an enhancer in SC, as evidenced in transgenic mice, but they also suggest that it mediates a different function in SC as compared to OL. We also conclude that our system represents a model to study the regulation of this SC enhancer (SCE) because it partially parallels the description revealed *in vivo* in transgenic mice.

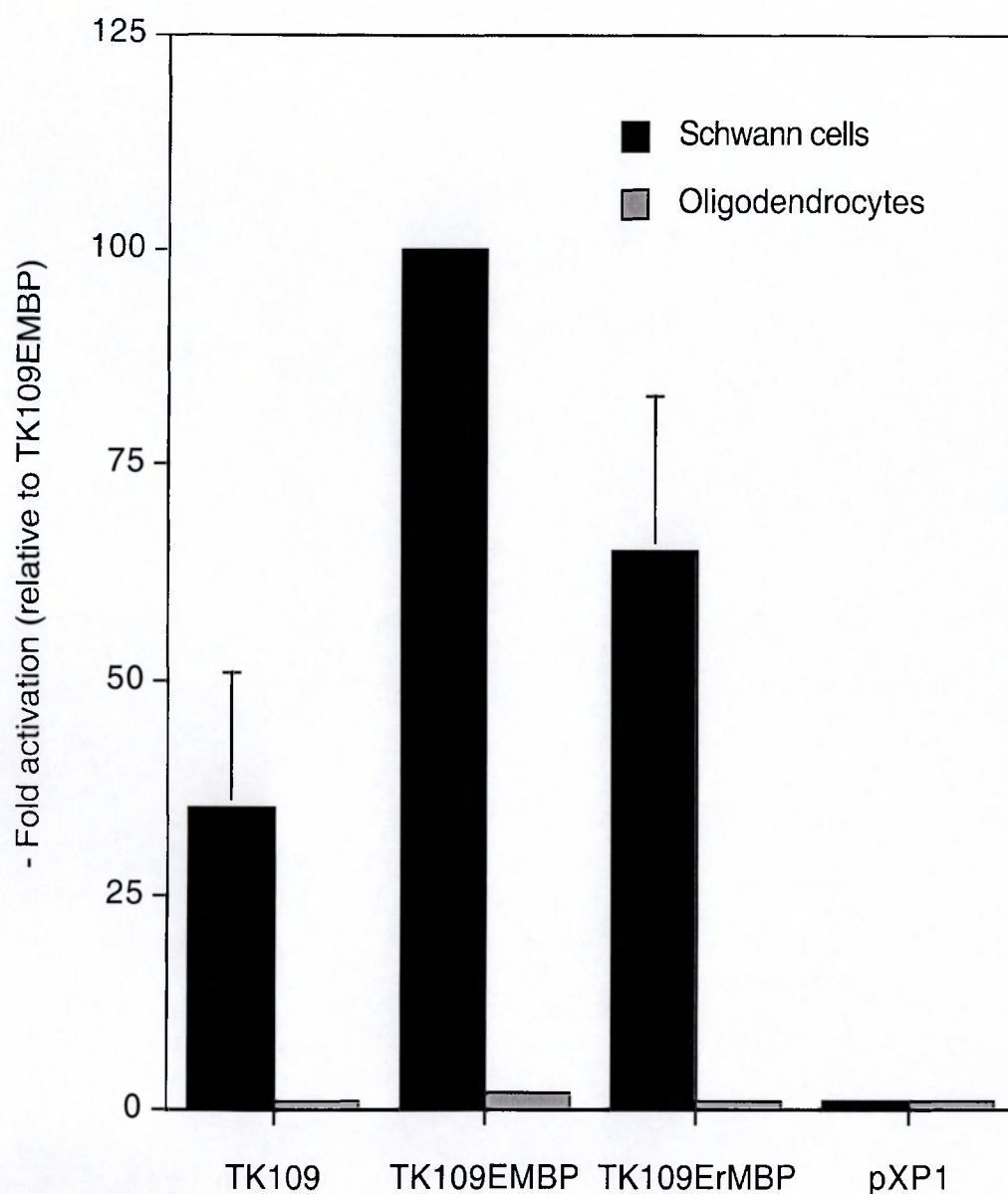


**Figure 2.4a**

The 588nt were cloned in both orientations upstream of the minimal homologous MBP promoter and transiently transfected in primary OL. The activity is relative to 256EBMP = 100. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

**Figure 2.4b**

The 588nt were cloned in both orientations upstream of the 1.1Kb region of the heterologous myelin P0 promoter and tested in primary SC. The activity is relative to P0Er = 100. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

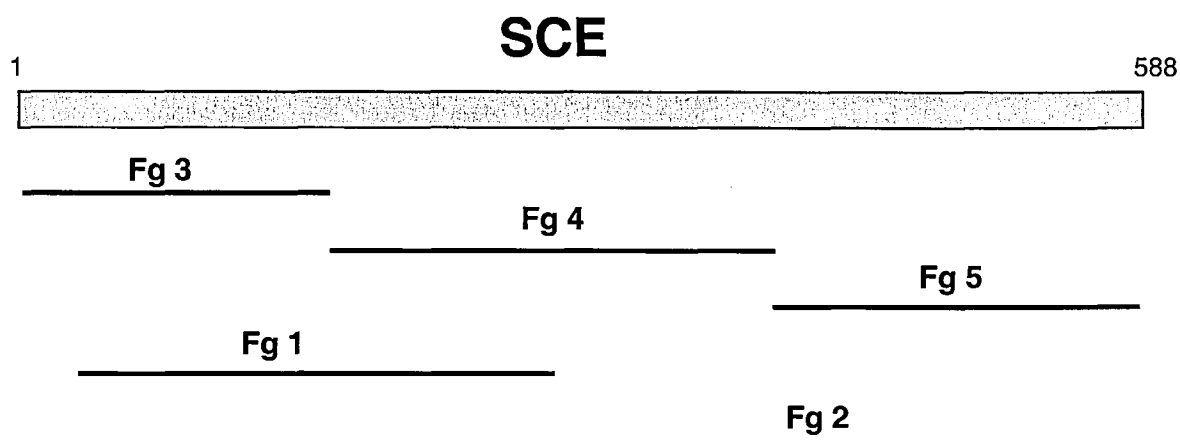


**Figure 2.4c**

The 588nt were cloned in both orientations upstream of the 109nt of the heterologous Thymidine Kinase promoter (from -109nt to +52nt) and tested both in primary OL and SC. The activity is relative to TK109EMBP = 100 transfected in primary SC. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

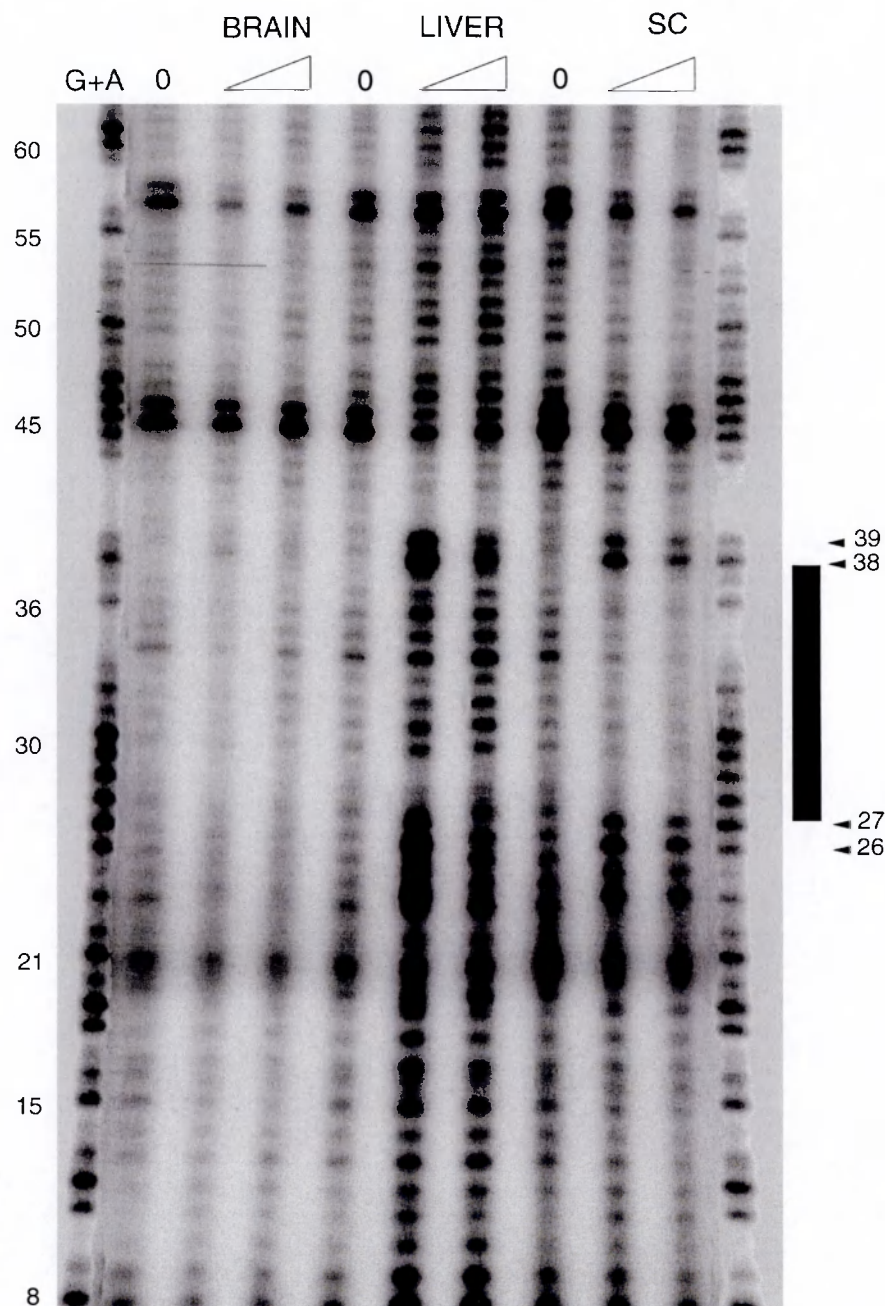
### BIOCHEMICAL ANALYSIS

Based on the analysis performed in transient transfection assays, we focused our attention on the SCE in order to identify regions inside it that are responsible for its differential behaviour, in SC as compared to OL. The presence of endogenous factors binding to SCE was tested by DNase I footprinting experiments. The 588nt were subdivided into five overlapping fragments: Fragment 1 from nucleotide 72 to nucleotide 290; Fragment 2 from nucleotide 290 to nucleotide 570; Fragment 3 from nucleotide 1 to nucleotide 195; Fragment 4 from nucleotide 195 to nucleotide 390; Fragment 5 from nucleotide 390 to nucleotide 588 (Fig. 2.5a). Increasing amounts of nuclear extracts prepared from either rat SC, mouse brain or liver, the last one used as a negative control, were incubated with end-labelled probes corresponding to SCE fragments, subsequently digested with DNase I and then resolved on a sequencing gel. We analysed both upper and lower strands of each fragment. We found regions specifically protected by each extract: regions protected by SC and brain but not by liver; and regions protected only by SC or only by brain. In particular we found four regions that were exclusively protected in SC nuclear extracts: Region 1 from nucleotide 33 to nucleotide 42; Region 2 from nucleotide 112 to nucleotide 127; Region 3 from nucleotide 372 to nucleotide 380; Region 4 from nucleotide 549 to nucleotide 554 (Fig. 2.5c). An example of one of these footprinting experiments is shown in Fig. 2.5b.



**Figure 2.5a**

Schematic representation of SCE overlapping fragments generated by restriction enzyme cuts and tested in DNase I footprinting analyses. Both upper and lower strands of each fragment were tested by DNase I digestion.

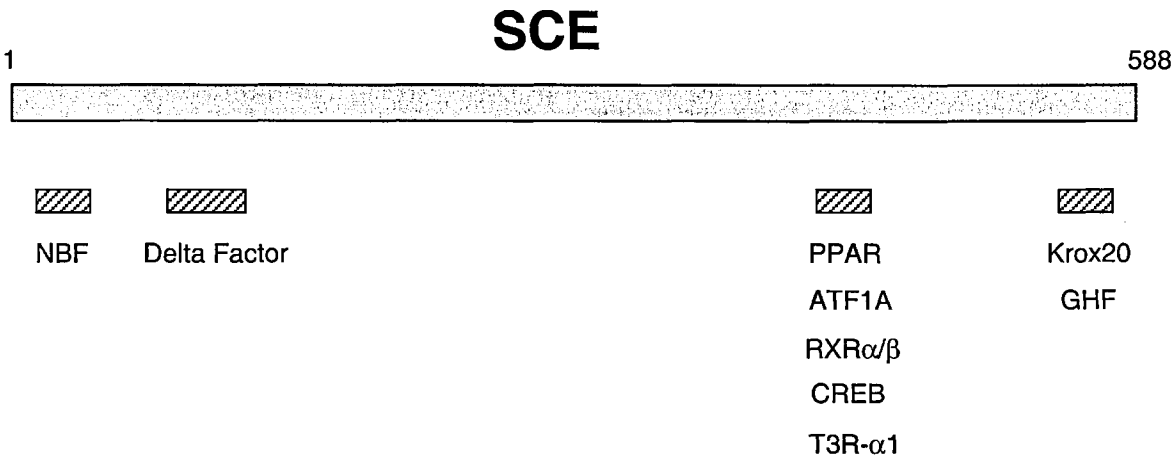


**Figure 2.5b**

DNase I footprinting analysis of the lower strand of fragment 3. The filled rectangle represents a protection present only in SC and not in brain or liver. The arrowheads indicate hypersensitive sites that delimit this protection in SC nuclear extracts. The same protection was confirmed also on the upper strand. Note that in liver, hypersensitive sites are present also at 38/39, but the protected region extends up to nucleotide 45. G+A: Maxam and Gilbert sequence reaction; 0: no nuclear extract.

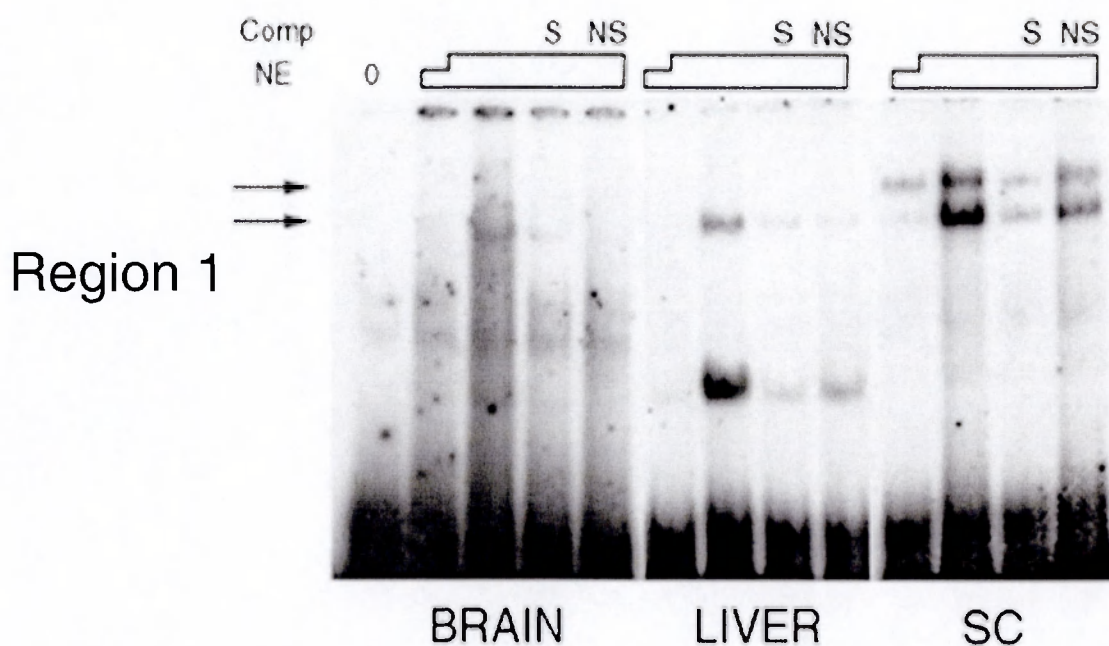
We focused our attention on these SC-specific regions because we wanted to better define the binding responsible for SCE activity. Therefore we searched for known transcription factors that can bind to these protected sequences using a TransFac database ([www.cbil.upenn.edu/cgi-bin/tess/tess33](http://www.cbil.upenn.edu/cgi-bin/tess/tess33)) and we found the possible binding sites for some of them, including Nonamer Binding Protein (NBF), Activating Transcription Factor 1A (ATF), cAMP Responsive Element Binding site (CREB), Retinoid X Receptor (RXR $\alpha/\beta$ ), Thyroid Hormone Receptor (T3R- $\alpha$ 1), Growth Hormone Factor (GHF). In particular we found the putative binding site for Peroxisome Proliferator-Activated Receptor (PPAR), a family of transcription factors known to be involved in the regulation of genes mediating lipid metabolism and recently identified also in OL (Granneman *et al.*, 1998) (Fig. 2.5c). Just upstream of the last region differentially protected at the 3' end of the enhancer, there is a consensus-binding site for Krox-20 transcription factor. Krox-20 is particularly interesting, as it is required to activate myelination in the peripheral nerve, as shown in Krox-20 null mice (Topilko *et al.*, 1994). However, the region containing the Krox-20 binding site was not sufficient to replicate SCE activity either in vivo or in vitro. In fact, this element is maintained in the 8.4Kb construct and this plasmid is not sufficient to drive the expression of a reporter gene in either transgenic mice, or in transfected SC. To determine whether this element is necessary, Forghani and colleagues mutated this site in one transgenic line. They showed that despite this mutation, the *lacZ* gene is expressed throughout the PNS, demonstrating that it is not necessary to confer the specific expression in SC (Forghani *et al.*, 2001). Thus, the Krox-20 binding site is neither necessary nor sufficient for SCE activation.





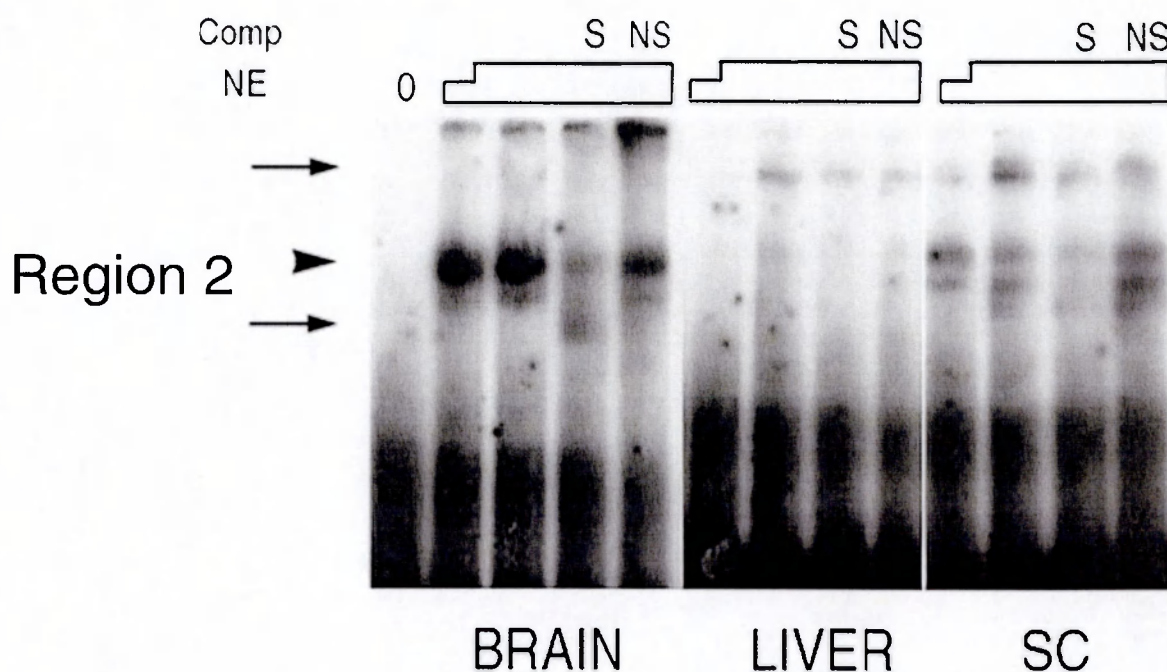
**Figure 2.5c**  
Schematic representation of protected regions identified by DNase I footprinting analyses only in SC nuclear extracts (striped boxes). Under each box the consensus binding sites identified by TransFac analyses are indicated.

To confirm the differential binding and explore the cellular origin of binding proteins previously identified, we designed oligonucleotides encompassing the regions protected only in SC nuclear extract and we tested for their ability to bind SC, brain and liver nuclear extracts in Electro Mobility Shift Assay (EMSA). This analysis confirmed that in all regions the binding was different in SC as compared to brain and liver (Fig.2.6a, 2.6b, 2.6c, 2.6d). Because in brain only a proportion of the cells are OL, we decided to repeat the same assays using nuclear extracts prepared from CG4 cells, a rat oligodendroglial population (Louis *et al.*, 1992). In these analyses we found that in region 1, 2 and 4 the binding was different (Fig.2.7a, 2.7b, 2.7c). Taken together these results indicate that, at least in three regions, there is a differential set of trans-activating factors in SC as compared to OL, in large part confirming the results obtained in the DNase I footprinting assays. Moreover the presence of this set of binding proteins specifically present in SC and different from OL, may explain the differential function of SCE in SC and in OL.



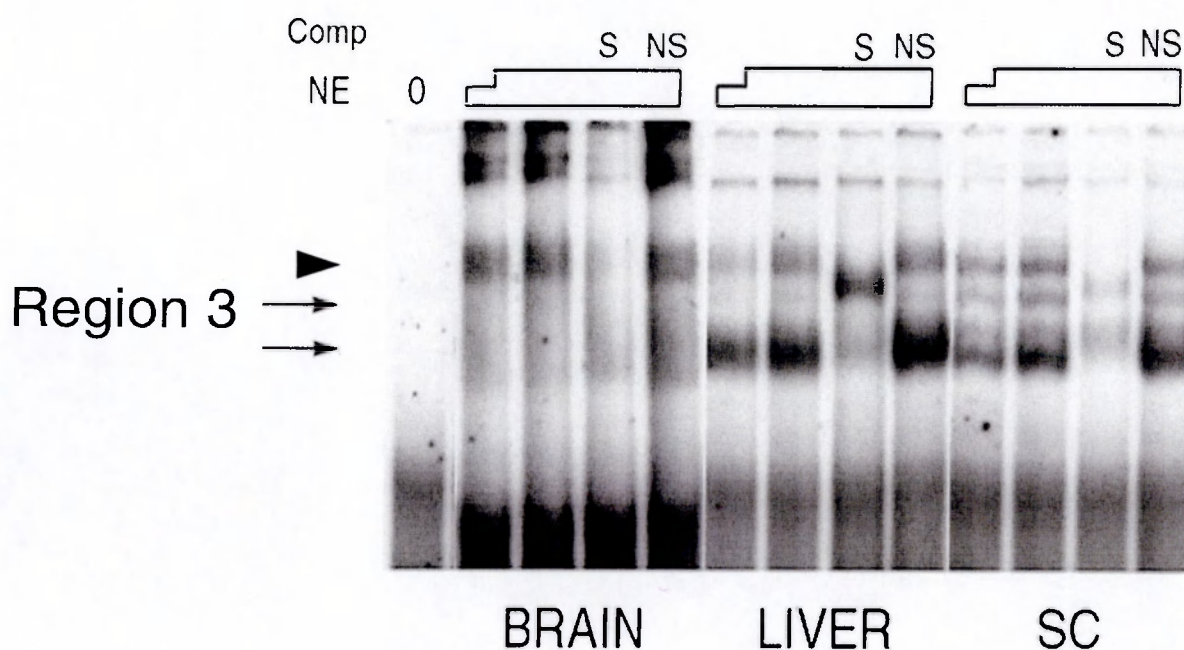
**Figure 2.6a**

EMSA analysis with probe encompassing region 1 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from brain, liver and SC. Bindings were specifically competed out using the same regions 100x in excess as cold competitor (S). They were not competed out by unrelated cold oligonucleotide 100x in excess (NS). Black arrows denote bindings specific for SC.



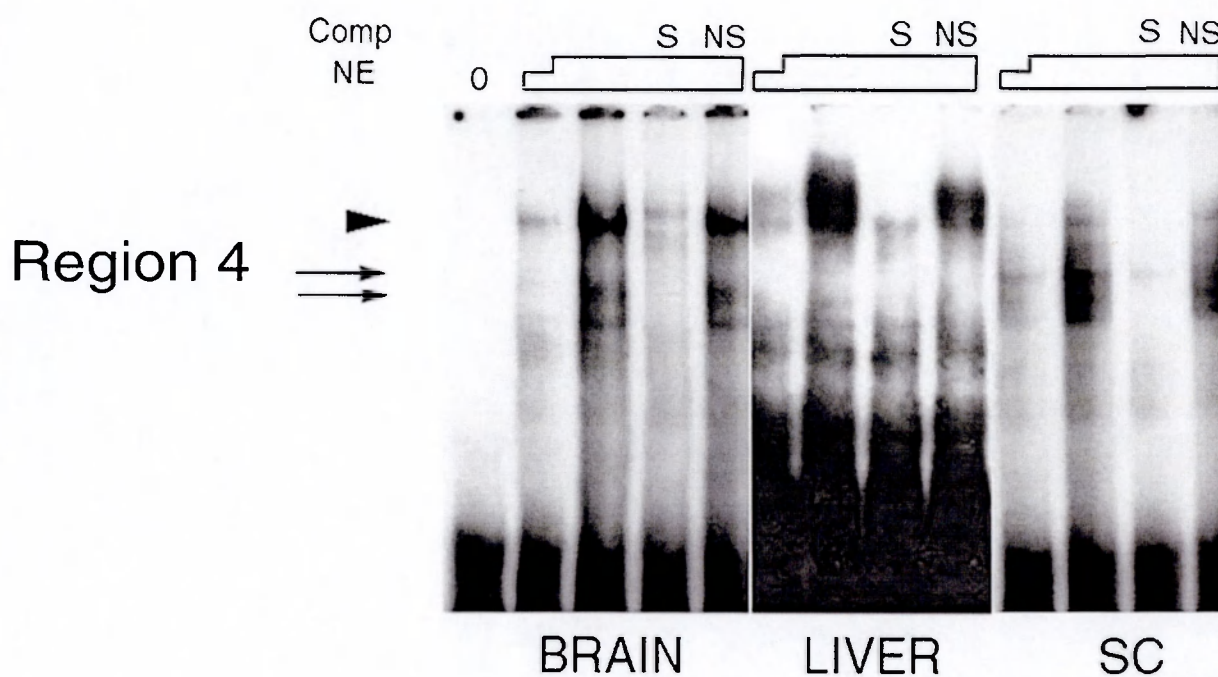
**Figure 2.6b**

EMSA analysis with probe encompassing region 2 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from brain, liver and SC. Bindings were specifically competed out using the same regions 100x in excess as cold competitor (S). They were not competed out by unrelated cold oligonucleotide 100x in excess (NS). Black arrows denote bindings specific for SC, while arrowheads indicate bindings present both in SC and brain.



**Figure 2.6c**

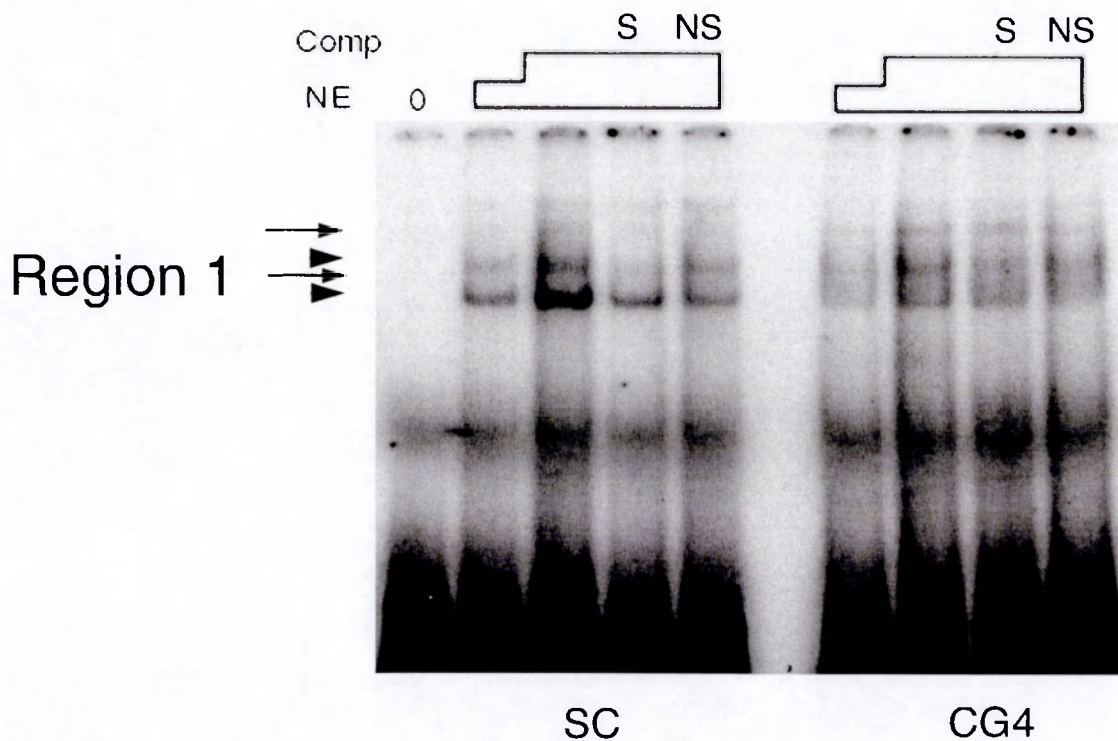
EMSA analysis with probe encompassing region 3 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from brain, liver and SC. Bindings were specifically competed out using the same regions 100x in excess as cold competitor (S). They were not competed out by unrelated cold oligonucleotide 100x in excess (NS). Black arrows denote bindings specific for SC, while arrowheads indicate bindings present both in SC and brain.



**Figure 2.6d**

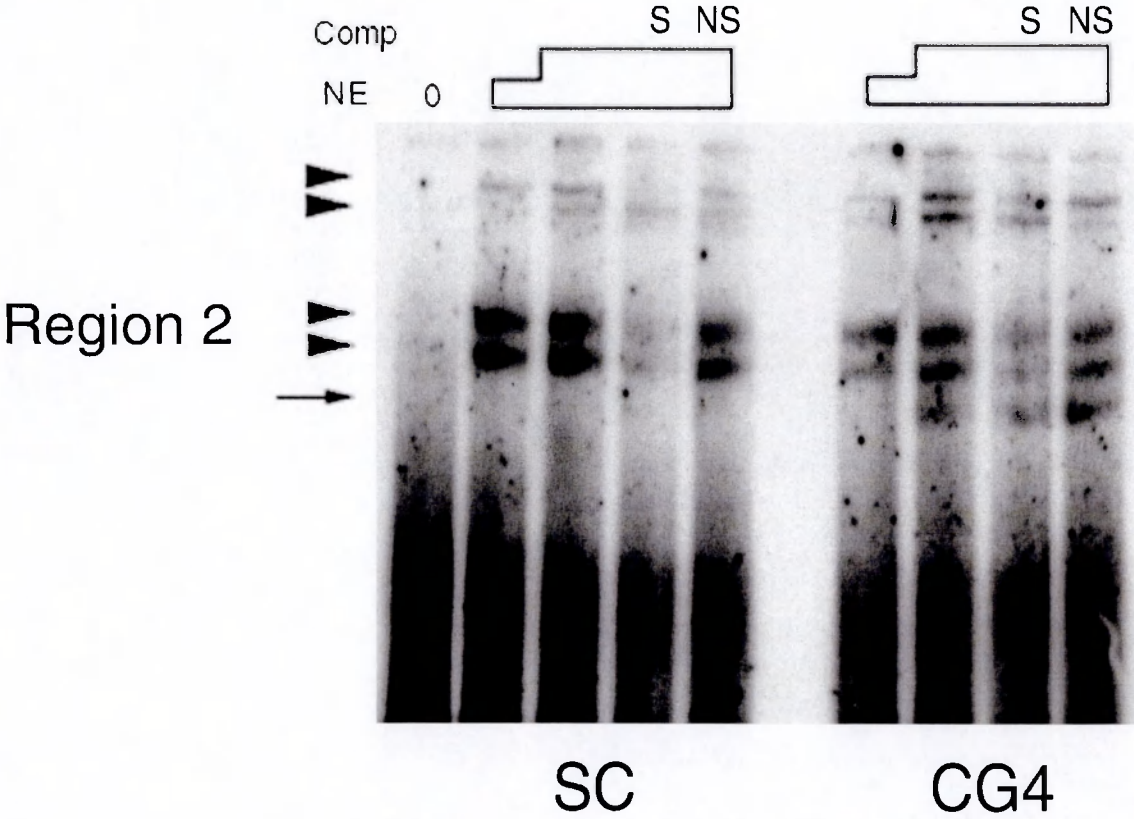
EMSA analysis with probe encompassing region 4 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from brain, liver and SC. Bindings were specifically competed out using the same regions 100x in excess as cold competitor (S). They were not competed out by unrelated cold oligonucleotide 100x in excess (NS). Black arrows denote bindings specific for SC, while arrowheads indicate bindings present both in SC and brain.





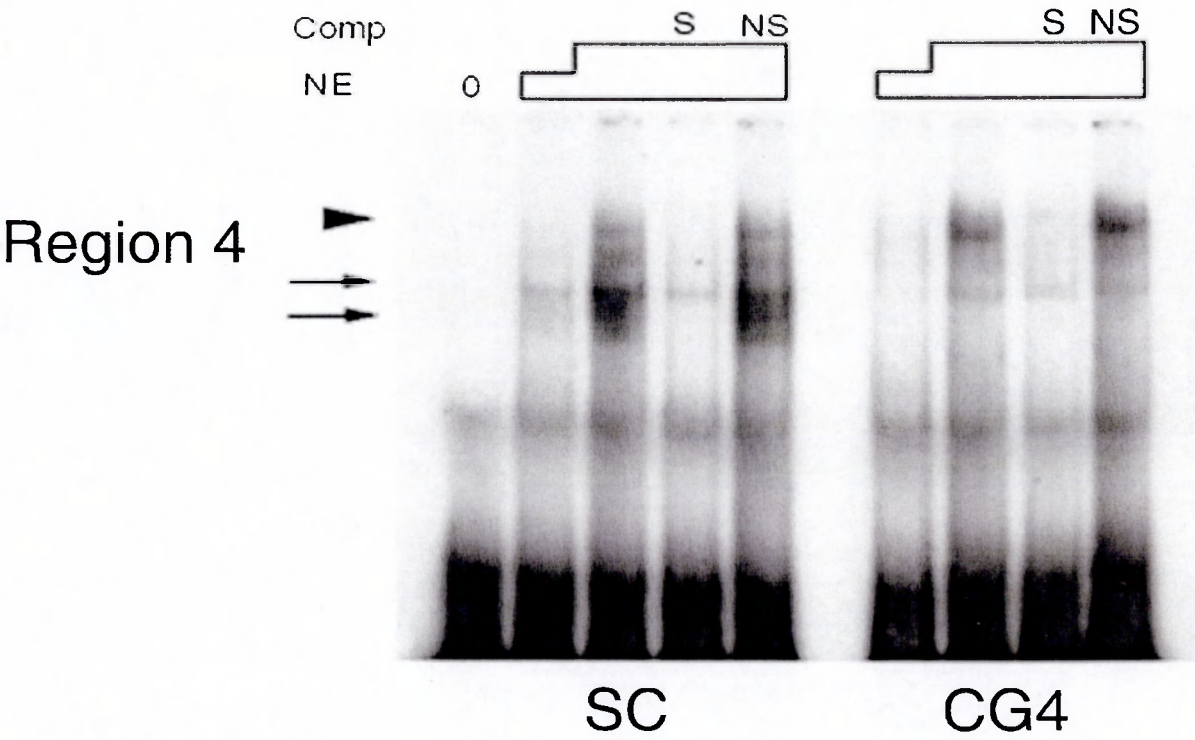
**Figure 2.7a**

EMSA analysis with probe encompassing region 1 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from SC and pure differentiated oligodendrocytes (CG4). The bindings were specifically competed out using the same region as a cold competitor 100x in excess (S), while unrelated oligonucleotide (NS) used 100x in excess is not able to compete out the binding. Black arrows denote bindings specifically present only in SC, while the arrowhead denote a binding present both in SC and CG4.



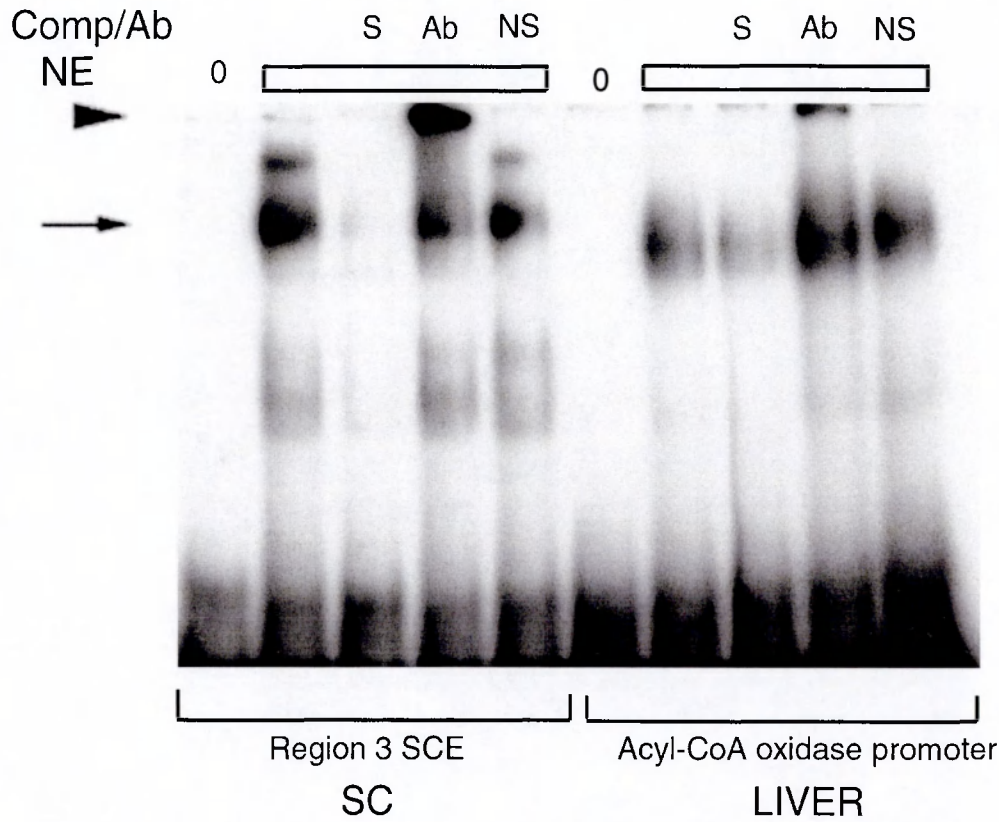
**Figure 2.7b**  
 EMSA analysis with probe encompassing region 2 differentially protected in SC. Probe was tested with 0-4 µg of nuclear extracts prepared from SC and pure differentiated oligodendrocytes (CG4). The bindings were specifically competed out using the same region as a cold competitor 100x in excess (S), while unrelated oligonucleotide (NS) used 100x in excess is not able to compete out the binding. Black arrows denote bindings specifically present only in SC, while arrowheads denote a binding present both in SC and CG4.





**Figure 2.7c**  
 EMSA analysis with probe encompassing region 4 differentially protected in SC. Probe was tested with 0-4  $\mu$ g of nuclear extracts prepared from SC and pure differentiated oligodendrocytes (CG4). The bindings were specifically competed out using the same region as a cold competitor 100x in excess (S), while unrelated oligonucleotide (NS) used 100x in excess is not able to compete out the binding. Black arrows denote bindings specifically present only in SC, while arrowheads denote a binding present both in SC and CG4.

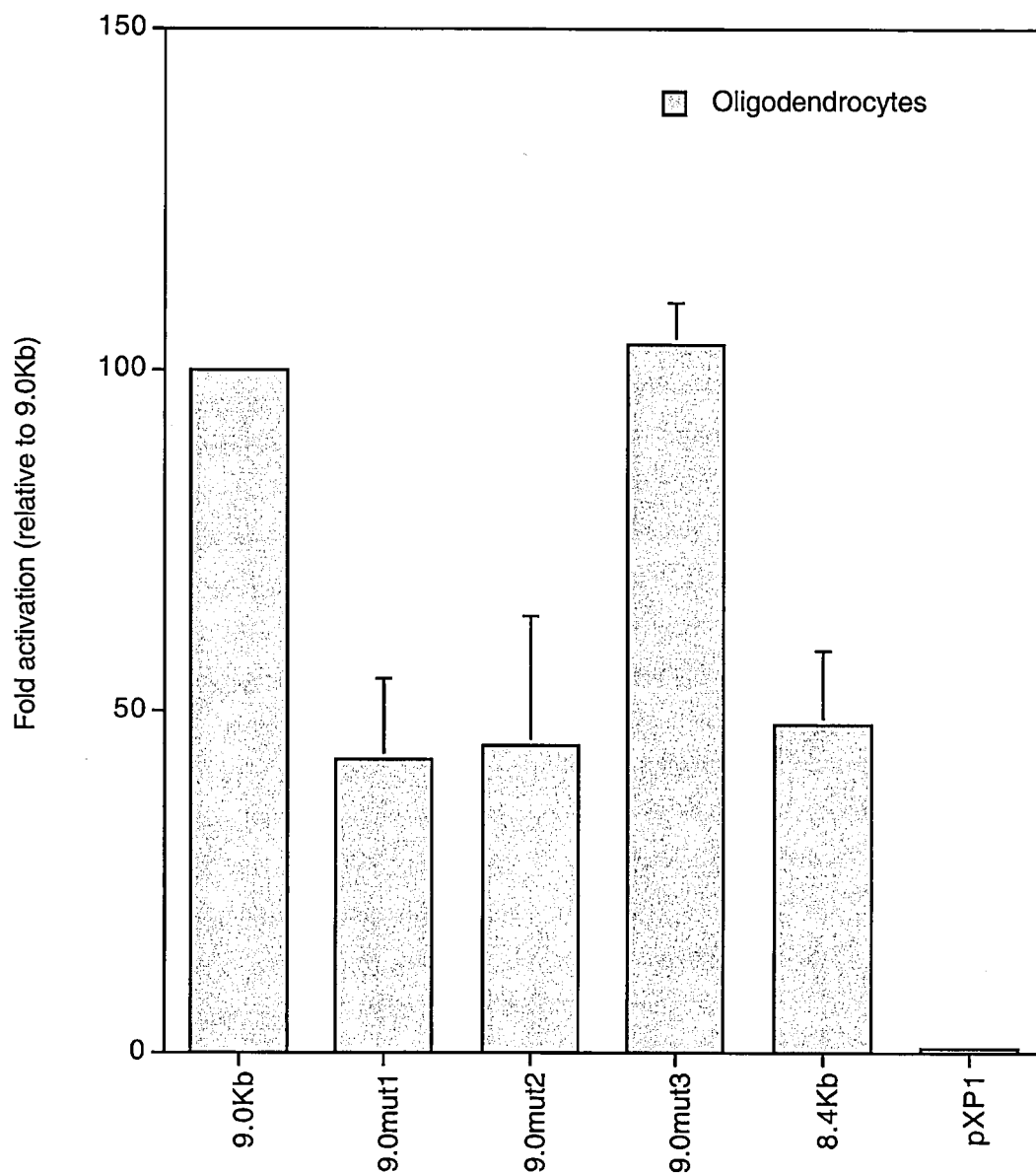
The use of specific antibody can help in the identification of proteins that form a binding complex in an EMSA assay. In fact if an antibody recognises a protein present in the complex, when added to the binding reaction, it changes the mobility of one or more of the retarded bands visualised in the acrylamide gel. To verify one prediction of the TransFac analysis for a protein relevant in myelination, we performed EMSA supershift analysis. We used an antibody able to recognise all PPAR isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). Using nuclear extracts prepared from SC we changed the mobility of a band in the complexes bound to region 3, in which we previously found the putative binding site for PPAR (Fig. 2.8). As positive control, we used the Acyl-CoA oxidase promoter region that was previously shown to contain a PPAR responsive element binding site that can be supershifted by an antiserum directed against the same family of transcription factor (Forman *et al.*, 1997).



**Figure 2.8**

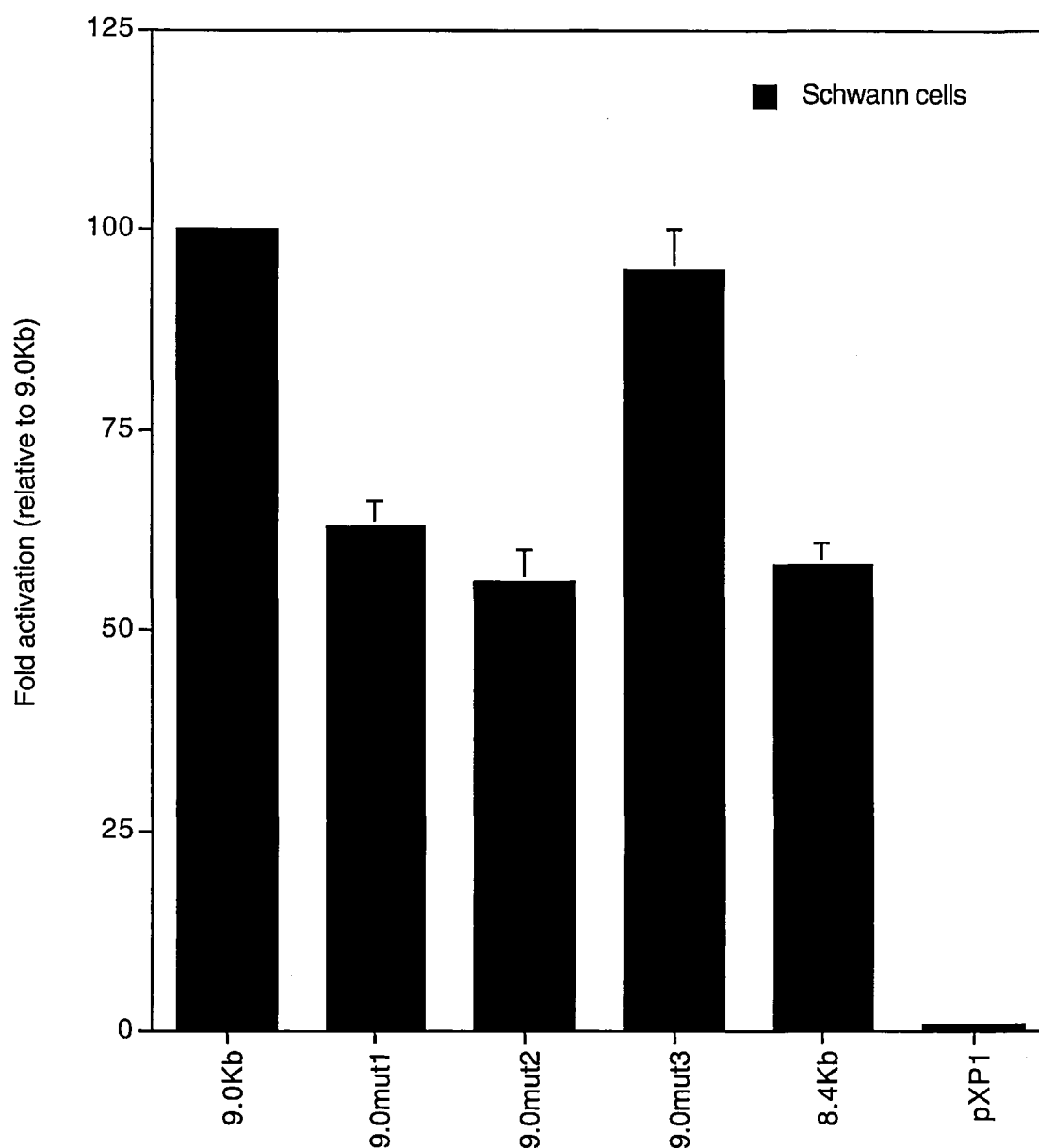
EMSA supershift analysis with probe encompassing region 3 differentially protected in SC. 0-8µg of nuclear extracts prepared from SC were used to bind a probe containing the putative binding site for PPAR family of transcription factor. The binding was specifically competed out using the same region as a cold competitor 100x in excess (S), while unrelated oligonucleotide, used 100x in excess (NS), is not able to compete it out. The use of an antibody able to recognize all PPAR isoforms ( $\alpha, \beta/\delta, \gamma$ ) can specifically supershift a band in SC nuclear extracts (arrowhead). The black arrow indicates PPAR binding that is present also in liver. The same result is shown in liver nuclear extract using a probe designed to the single Peroxisome Proliferator Response Element (PPRE) of the Acyl-CoA oxidase.

To test whether the differentially protected regions identified in SC have a functional role in regulating SCE activity, we specifically mutated them by transversion in the context of the 9.0Kb promoter. In particular we transverted region 1 (10nt), 2 (16nt) and 3 (9nt). We did not transvert region 4 (Krox-20) because it was already shown that it is neither sufficient nor necessary to direct the expression of the reporter gene in SC. We mutagenised each region by PCR using appropriately designed oligonucleotides. Each mutation was confirmed by sequence analysis and re-cloned in the native context of the 9.0Kb construct. We transiently transfected these mutated constructs together with 9.0Kb and 8.4Kb constructs in primary cultures of OL and SC. We compared the luciferase activity of these mutations relative to the 9.0Kb as 100%. Transient transfection analyses in primary OL revealed that transversion of region 1 reduced the activity by approximately 60% and mutation in region 2 by approximately 50%. On the contrary, mutation in region 3, which contains the PPAR binding site, had no effect (Fig. 2.9a). Transient transfections of these plasmids in primary SC revealed that mutation of region 1 reduced luciferase activity by approximately 40% whereas mutation of region 2, as in OL, reduced the activity by approximately 50%. Surprisingly mutation in the consensus-binding site for PPAR did not have any effect (Fig. 2.9b).



**Figure 2.9a**

Transient transfection analysis in primary OL of plasmids containing mutations in three regions differentially protected in SCE. The final activity is relative to 9.0Kb = 100. Mutations in regions 1 and 2 reduce the activity by approximately 50%, while mutation in region 3, containing the PPAR binding site, had no significant effect. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.



**Figure 2.9b**

Transient transfection analysis in primary SC of plasmids containing mutations in three regions differentially protected in SCE. The final activity is relative to 9.0Kb = 100. Mutations in regions 1 and 2 reduce the activity by approximately 50%, while mutation in region 3, containing the PPAR binding site, had no significant effect. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

To further dissect the SCE region responsible for the enhancer activity, we created a series of internal 100bp deletions and we transiently transfected them into primary cultures of OL and SC. This strategy had two advantages over the previous analysis: first it was random and made no assumptions about the specificity of nuclear extract binding, and second it increased the probability that cooperative cis-acting elements would be removed together. We generated these deletions using unique single restriction enzymes sites present in the MBP enhancer or, when this strategy was not possible, we created them through standard PCR technique using appropriately designed oligonucleotides. We named these deletions  $\Delta a$ ,  $\Delta b$ ,  $\Delta c$ ,  $\Delta d$ ,  $\Delta e$ ,  $\Delta f$  (Fig. 2.10a). In particular:

$\Delta a$  comprises deletion of the first 103nt, starting from SacII site to BstEII site;

$\Delta b$  comprises deletion from 103 to 204, starting from BstEII site to StuI site;

$\Delta c$  comprises deletion from 204 to 290, starting from StuI site to BglII site;

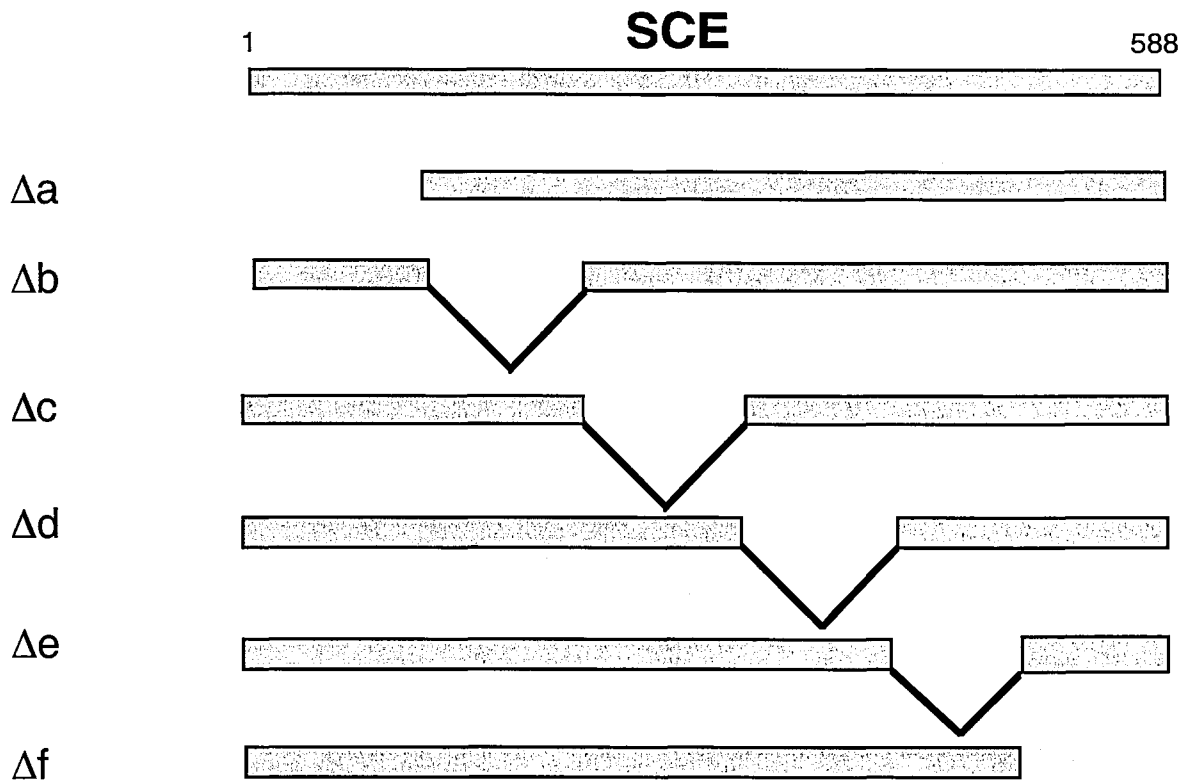
$\Delta d$  comprises deletion from 290 to 400, and it was generated by PCR;

$\Delta e$  comprises deletion from 400 to 519, and it was generated by PCR;

$\Delta f$  comprises deletion from 519 to 588, starting from NaeI site to SacI site.

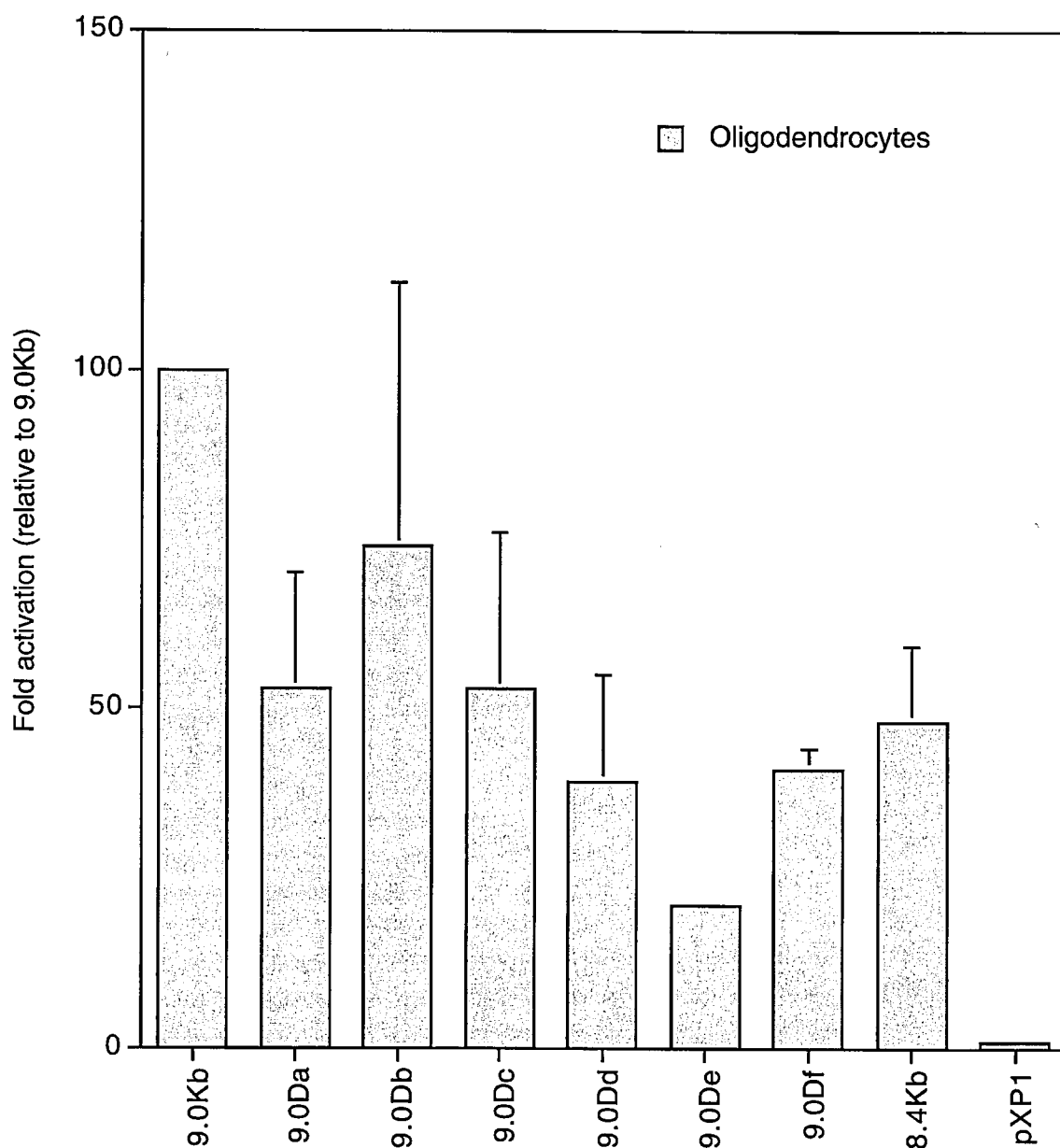
Both  $\Delta d$  and  $\Delta e$  were confirmed by sequence analysis. Once generated all constructs were transiently transfected into primary cultures of OL and SC and they were analysed relative to 9.0Kb and 8.4Kb constructs. Both in OL (Fig. 2.10b) and in SC (Fig. 2.10c) we found that most deletions showed a trend toward reducing activity of luciferase but the results were not statistically significant. The only region that seems to be clearly relevant from this analysis is  $\Delta e$  whose deletion reduces the activity of the reporter gene approximately 65% in SC and 80% in OL. Consistent with the observation that this region is relevant both for SC and OL, previous biochemical analysis showed that it is not differentially protected in EMSA analysis of these two cell types.

All together, the data of site-direct mutants and random mutants in SCE suggest that activation depends on multiple sites and that activation in these cell culture models is too low to clearly identify specific cis-acting elements.



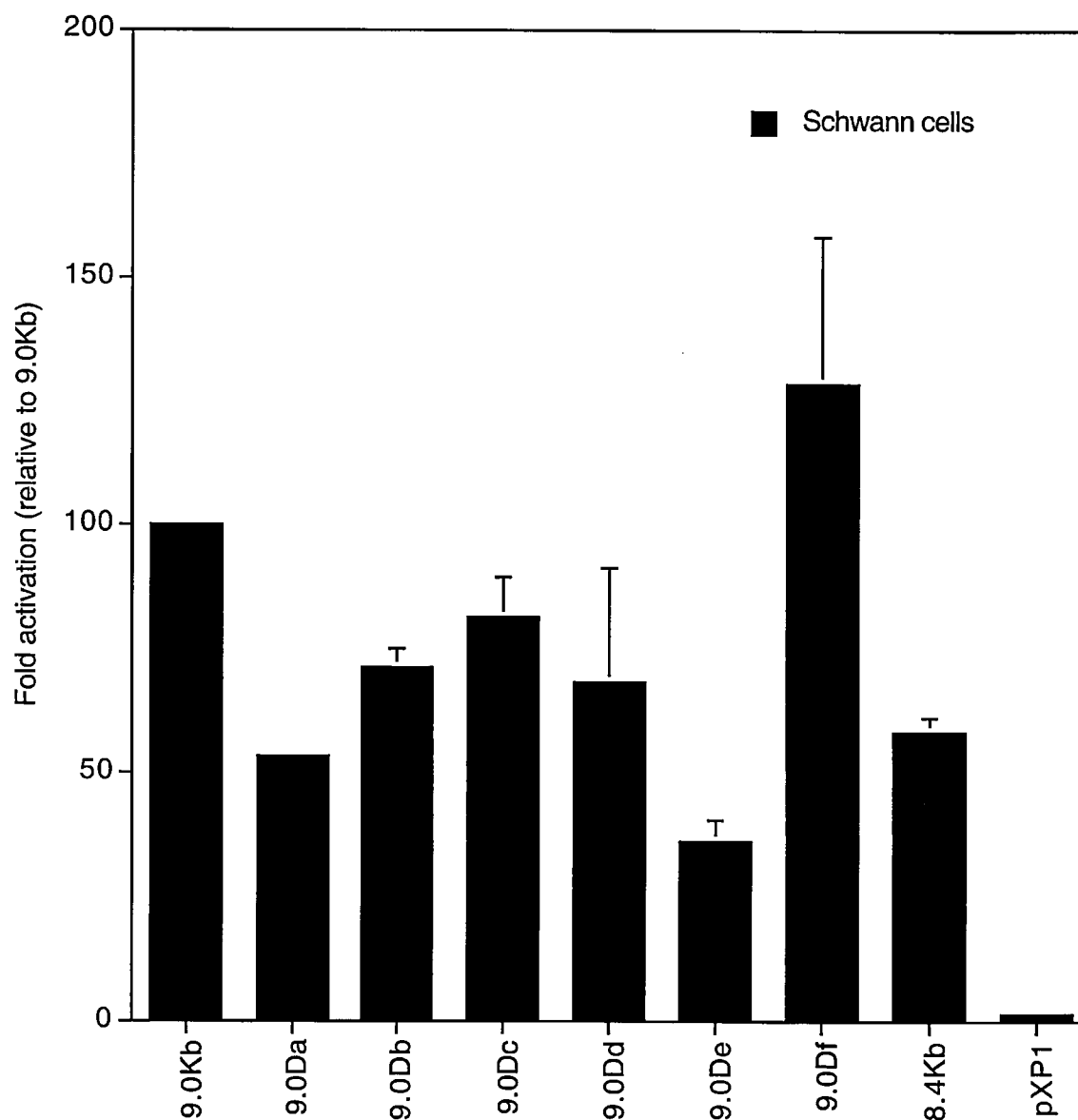
**Figure 2.10a**  
Schematic representation of SCE internal deletions. Deletions were generated using unique restriction enzyme sites or standard PCR technique using specific oligonucleotides.





**Figure 2.10b**

Transient transfection analysis in primary OL of plasmids containing SCE internal deletions. The final activity is relative to 9.0Kb = 100.  $\Delta e$  deletion that removes from 400nt to 519nt, reduces the activity by approximately 60%. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.



**Figure 2.10c**

Transient transfection analysis in primary SC of plasmids containing SCE internal deletions. The final activity is relative to 9.0Kb = 100.  $\Delta e$  deletion that removes from 400nt to 519nt, reduces the activity by approximately 60%. Bars represent the mean luciferase activity  $\pm$  S.E.M. of at least three independent experiments.

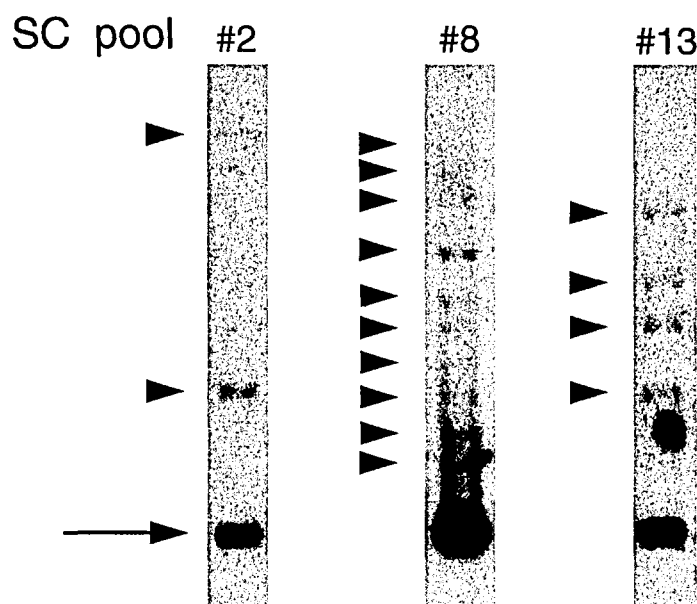
## 2.2 ANALYSIS OF SCE IN STABLY TRANSFECTED SC

Transient transfection experiments in primary SC showed that SCE behaves as an enhancer not only in transgenic mice but also in vitro. Thus SCE could specifically direct the activity of a reporter gene in an orientation and distance independent way and transactivate a heterologous promoter as well as myelin promoters. However the amplitude of activation is not as high as would be expected based on transgenic mice. Forghani and colleagues showed, in multiple independent lines, that SCE activated robust *lacZ* expression above an undetectable background in the absence of SCE. In our experience (Taveggia and colleagues, 1998), with other *lacZ* transgenes in the nervous system, such a change in staining represents at least 100-fold induction. Instead the largest activation we revealed in SC cultured was 3-fold. Given that the cis-acting sequences utilized in transgenic mice and transfected SC are identical, we are probably lacking some element acting in trans on SCE. The first possibility is that this reduced activation is due to the episomic state of the plasmid. Chromatin can actively regulate the expression of a promoter-reporter construct and therefore the episomic state may not exactly recapitulate the regulation pattern. This was previously confirmed also for another myelin gene, the PLP gene (Nave and Lemke, 1991).

To investigate the role of chromatin in regulating SCE we produced stably transfected SC. In particular we created stable transfectants for 9.0Kb, 8.4Kb, 3.1Kb and the promoterless construct pXP1. To select stably transfected SC each plasmid was co-transfected with pMAMneo plasmid containing the *neo* gene for positive selection. To average insertions near unrelated transcriptional regulatory sequences that could alter SCE activity, each plasmid was transfected into six different plates. After selection, we obtained a range of 10 to 20 clones per plate. The clones obtained in each plate were together pooled by passage, resulting in 4-6 independent pools of cells for each construct, each likely representing a different insertion site (see Table 1 and Fig. 2.11).

To confirm the polyclonal nature of the pools, and to normalise the luciferase activity determined by the biochemical assay, we performed Southern blot analysis on genomic DNA prepared from each single pool. We digested DNA with XbaI restriction

enzyme that produces a fragment of approximately 1.6Kb overlapping the luciferase gene. As probe we used a DNA fragment contained in the luciferase gene. This strategy allowed us to detect luciferase copy number as well as 3' junction fragments representing independent insertions. Fig. 2.11 shows that clones contained multiple insertions.



**Figure 2.11**

Southern blot analysis on SC pools #2 (pXP1), #8 (8.4Kb) and #13 (pXP1) obtained after G418 selection. Genomic DNA was digested with XbaI restriction enzyme. The probe used was the XbaI/BsrGI DNA fragment contained in the luciferase gene that allows also the recognition of 3' junction fragments. The arrow denotes the 1.6Kb fragment of the luciferase gene; whereas arrowheads indicate multiple integration sites.

In order to normalise the luciferase activity of the pools as measured by biochemical assay, we quantitated copy number by performing densitometry of total luciferase signal. We expressed this quantity relative to the signal obtained re-probing the Southern for a single copy autosomal gene, the P0 gene, using the BamHI/HindIII fragment overlapping P0 exon 1. The copy number obtained by this analysis for various pools of each construct is represented in Table 1.

TABLE 1

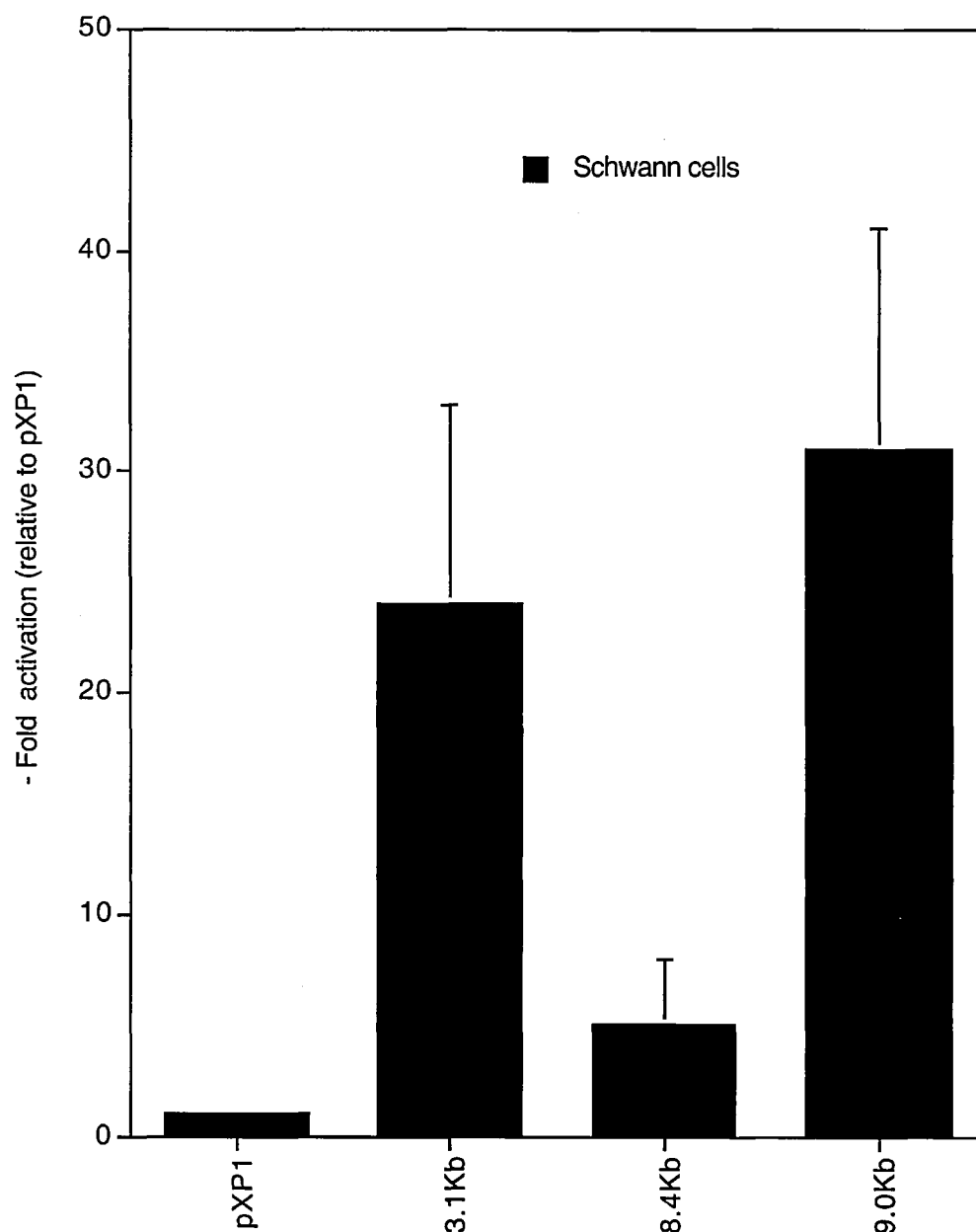
COPY NUMBER (luc signal/P0 signal x 2)

<u>Pool number</u> <u>Plasmid</u>						
pXP1	#1	#2	#3	#13	#14	#15
	11.6	11.4	2.4	11.6	2.6	9
3.1Kb	#4	#6	#17	#18		
	9.8	20	14	12.6		
8.4Kb	#8	#9	#19	#20		
	53	12.2	13.6	4.4		
9.0Kb	#10	#11	#22	#23	#24	
	2	8.6	2.8	2	6	

Table 1

Quantitation of copy number of the integrated luciferase gene after stable transfection in SC. The total luciferase signal, determined by densitometric analysis, is expressed relative to the signal obtained re-probing the same Southern blot, with a BamHI/HindIII fragment recognising the single autosomal P0 gene.

By doing this analysis we observed that the 9.0Kb construct increased the activity by approximately 30-fold, while the 8.4Kb was increased 5-fold compared to the promoterless construct (statistical analysis:  $p=0.005$ ). Thus the 9.0Kb activation was increased at least 6-fold above 8.4Kb. This represents a doubling of the increase seen by 9.0Kb over 8.4Kb in transient transfection analyses. This represents an activation of the 9.0Kb construct, and not repression of the other constructs, as the 8.4Kb was activated 5-fold above pXP1 in both transient and stably transfected SC. On the contrary, the 3.1Kb construct activity was paradoxically increased in both transient and stably transfected SC, even if the overall activation of 3.1Kb in the stably transfected was reduced (compare Fig. 2.3b with Fig. 2.12). This result suggests that a chromatin context activates SCE, but it is still not sufficient to explain the absence of two orders of magnitude activation seen in transgenic mice.



**Figure 2.12**

Luciferase activity of primary SC stably transfected with 9.0KB, the 8.4Kb, the 3.1Kb constructs and the promoterless pXP1. Fold activation is relative to the promoterless construct pXP1 = 1. Note that the 9.0Kb construct increases the activity approximately 30-fold above pXP1, whereas the 8.4Kb construct has the same activity observed in transient transfection analysis ( $p=0.005$ ).

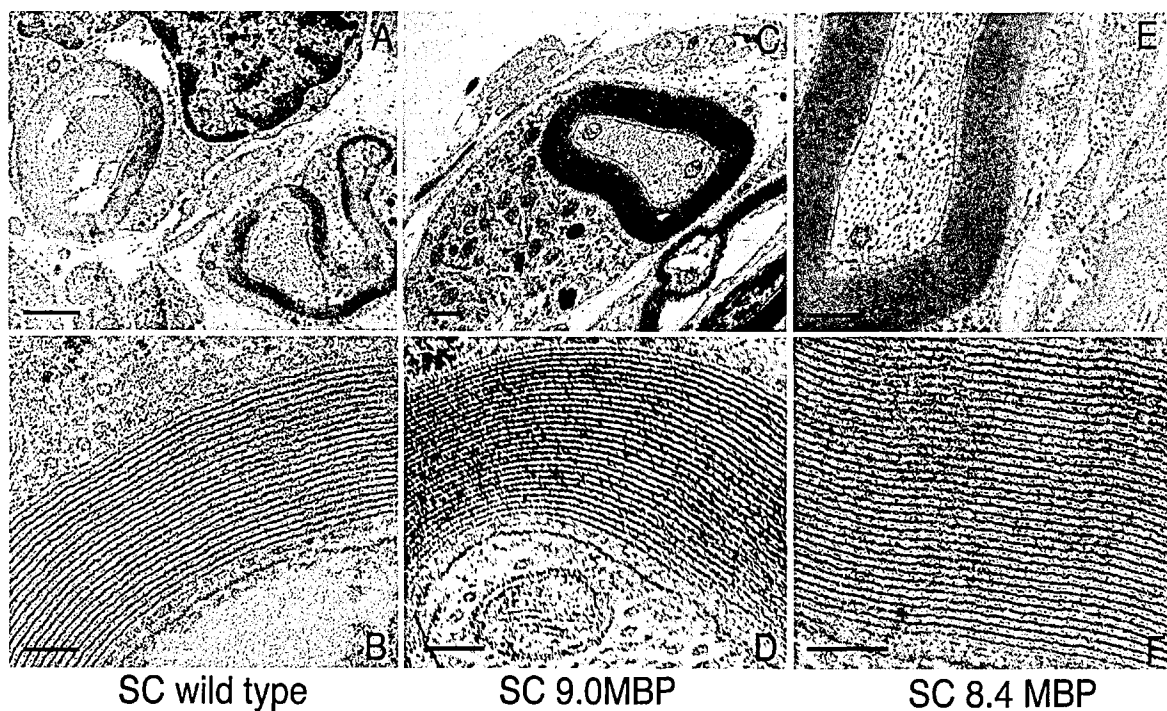
### 2.3 AXONAL INFLUENCE ON SCE MEDIATED-ACTIVITY

Another element that is well known to induce myelin gene expression is the axon. Axons upregulate MBP (or myelin gene) expression as suggested by analyses of transected nerves both in CNS and in PNS; 3-4 fold in optic nerve (Wrabetz *et al.*, 1998; Kidd *et al.*, 1990; McPhilemy *et al.*, 1990; Scherer *et al.*, 1992) and at least two orders of magnitude (P0) in sciatic nerve (Scherer *et al.*, 1994a).

To specifically test the effect of axons on the regulation of SCE, we set up a co-culture system in which previously characterised pools of stably transfected SC were put in contact with purified DRG neurons. We chose pools of SC that were in the middle range of copy number and luciferase activity near the mean value for that plasmid (see Table 1 and Fig. 2.12). In particular we chose pool # 24 for the 9.0Kb, pool #19 for the 8.4Kb, and pool #14 for the promoterless construct pXP1. The co-culture model is a well-developed and useful system through which it is possible to obtain myelination *in vitro* in a synchronised way. We prepared DRG neurons from rat at embryonic day 15.5 (E15.5), and we allowed them grow in the presence of nerve growth factor (NGF). Through addition of antimitotic agents, we reduced the number of fibroblasts and satellite cells present after purification. When the neurites reached an extensive expansion in the culture, either wild type or stably transfected SC were added back in order to repopulate the neurites. Addition of ascorbic acid stimulated SC to organise a basal lamina and initiate myelinogenesis.

To control for the capacity of our stably transfected SC to properly respond to axonal signals, we measured their ability to form compact myelin; myelinogenesis is in fact strictly axonally-dependent. Ultrastructural analyses performed on these SC-neuronal co-cultures showed that stably transfected SC for each construct myelinate as well as wild type SC. We checked myelin compaction after 17 days of ascorbic acid treatment, when many neurites are myelinated and we found that cells from each pool can form compact myelin (Fig. 2.13).





**Figure 2.13**

Ultrastructural analysis of SC-neuron co-cultures. In (A) and (B), neurites were re-populated with wild type SC. In (C) and (D) neurites were re-populated with SC stably transfected with 9.0Kb construct. In (E) and (F) neurites were re-populated with SC stably transfected with 8.4Kb construct. At high magnification (B), (D) and (F), myelin periodicity of compact fibers is normal both in wt, SC9.0MBP and 8.4MBP. Bars: (A) = 1µm; (B) and (D) = 0.140µm; (C) and (E) = 0.583µm and (F) = 0.116µm.

Normally, in co-culture experiments, SC are added back and allowed to re-populate the culture one week before the addition of ascorbic acid. During this period SC proliferate and sort axons in order to achieve a one to one relationship with them (Salzer and Bunge, 1980). In principal the activation of MBP promoter could be obtained by the simple contact of SC with neurites, without induction of myelination. The quantitative analysis of luciferase allowed us to distinguish between the requirement of SC-neurites contact or the necessity of the myelination process, because they occur in two distinct phases of the culture. To test this possibility we measured luciferase activity in 9.0Kb co-cultures, after SC seeding on neurites, and precisely 3 days before the addition of ascorbic acid (-d3). We found that axons, per se, are not sufficient to upregulate luciferase expression in this situation (Table 2). As a control we checked luciferase activity of the same SC seeded without neurons and we found that it is not significantly different from the one measured at -d3 (data not shown). Based on this set of experiments we conclude that the simple axonal contact, per se, is not sufficient to induce the expression of the MBP gene.

For this reason we induced the myelination process through ascorbic acid addition, and we tested luciferase activity of pools of stably transfected SC by biochemical assay. In particular we checked these time points: day 0 (d0), the day in which we induced myelination, after four days (d4), after 8 days (d8), after 12 days (d12) and after 15 days (d15). We monitored luciferase expression during the myelination process in all three types of stably transfected SC. We checked, at each time point, the activity of SC bearing the 9.0Kb construct and the pXP1 plasmid, as negative control. SC with the 8.4Kb construct were checked at d0 and after 15days of ascorbic acid treatment, when the myelination process is well extended, as evidenced by the number of MBP positive fibers (Fig. 2.14a). All results, expressed as a ratio between luciferase activity and amount of protein used, are summarised in Table 2. The measurements represent 2-5 independent co-culture experiments for most points. All values are expressed as the percentage of the peak value for 9.0MBP. We observed a small increase in luciferase activity in the 9.0Kb cultures, between d0 and d4, but only after d8 we saw an increase of approximately 3 fold, that remained stable until d15. Surprisingly the overall activation we observed in this system, measured with the biochemical assay, was not as strong as expected. On the

contrary luciferase activity in the pXP1 stable transfectants remained near background during all periods of myelination (Table 2).

**TABLE 2**

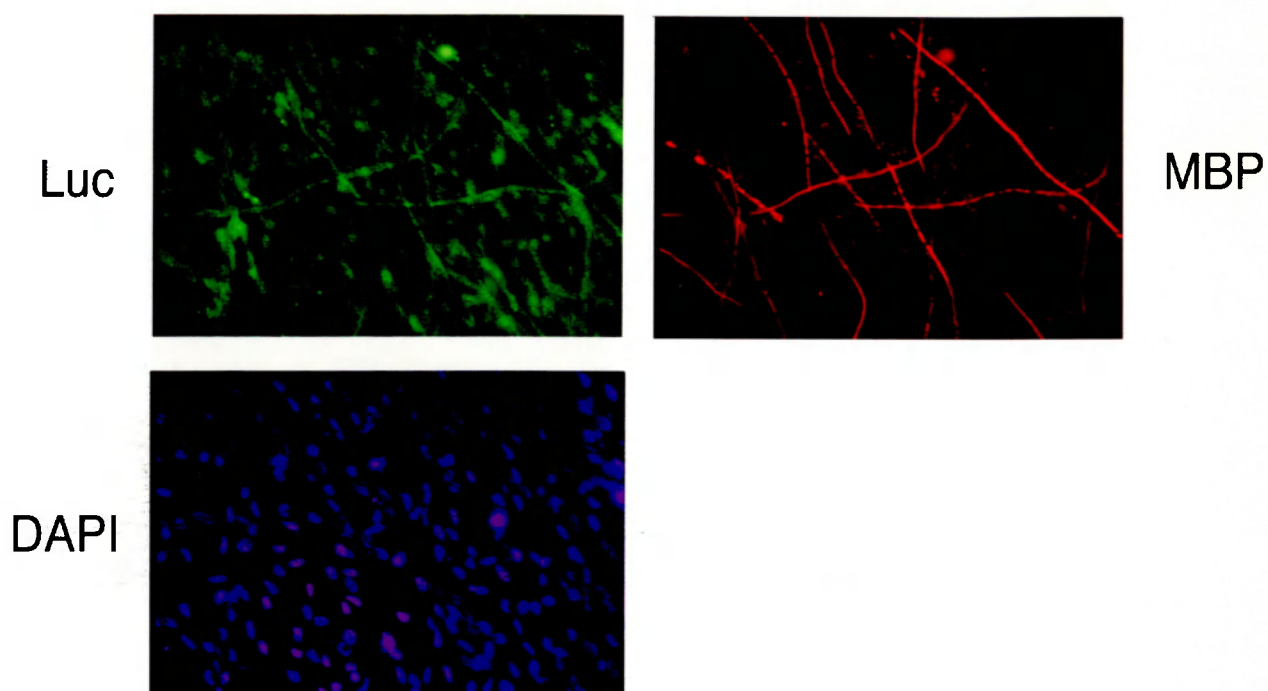
Luciferase specific activity (9.0MBP max activity=100)

	-d3	d0	d4	d8	d12	d15	8 days post cut at d6
SC 9.0MBP	17 $\pm$ 2	31 $\pm$ 7.5	50	100	100	100	37
SC 8.4MBP		24 $\pm$ 1.5		15		27 $\pm$ 0.5	
SC pXP1		3 $\pm$ 1		2 $\pm$ 1.5	1 $\pm$ 0.4	2 $\pm$ 1.1	

**Table 2**

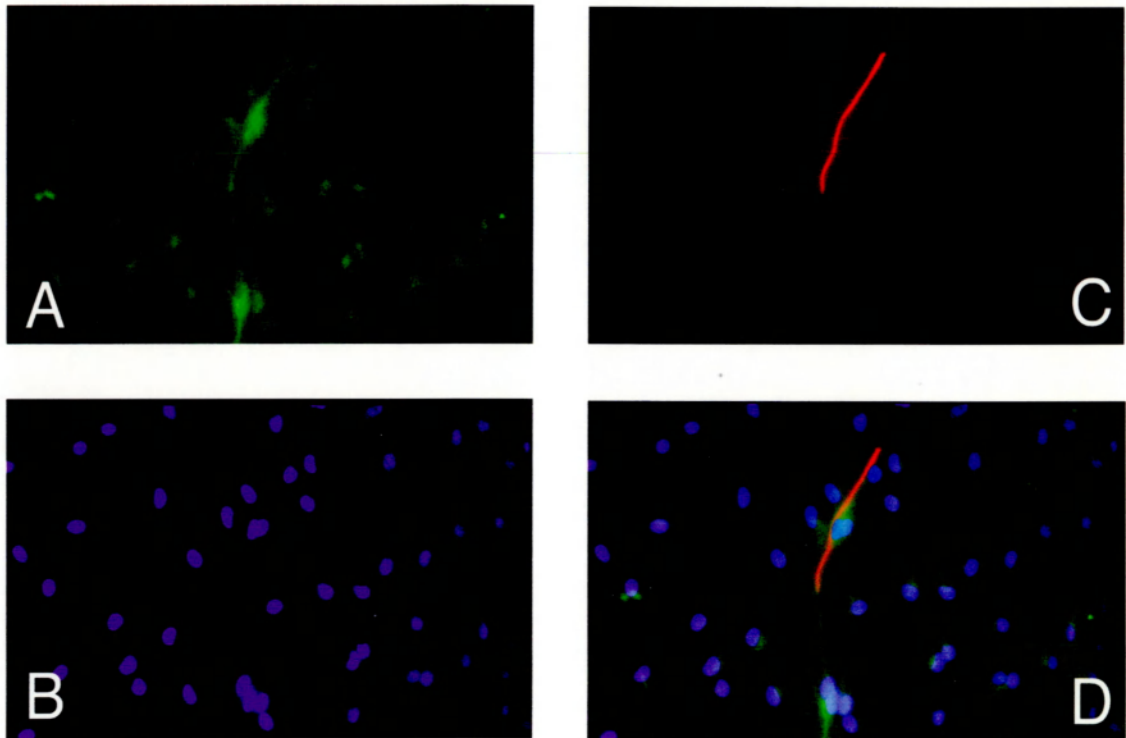
Luciferase activity of SC-neuron co-cultures at various time points before and after ascorbic acid addition. Neurites were re-populated with SC stably transfected with 9.0MBP, 8.4MBP, pXP1 constructs. Fold activation is measured as a ratio between luciferase value and amount of protein used in the assay. The final activity is relative to the 9.0MBP maximal activity detected as 100. Note the progressive increase in luciferase activity during myelinogenesis only in the 9.0MBP SC. In contrast luciferase activity measured on SC 9.0MBP 8 days after in vitro cut has an activity similar to d0.

One possible explanation for limited overall upregulation of luciferase in the 9.0Kb SC is activation in only some SC. To determine which SC activated luciferase, we performed immunofluorescence analyses on co-cultures containing 9.0Kb SC. We made a double staining using a polyclonal antibody against luciferase and a mouse monoclonal antibody against MBP, as a marker of myelination. We also used DAPI to visualise SC nuclei (Fig. 2.14a). We repeated these analyses several times on different sets of preparations, at the same time points used in the biochemical assay. Interestingly we observed at d8 an upregulation in luciferase activity but only in a restricted number of 9.0 SC (Fig. 2.14b). This is not due to a defect in the sensitivity of the antibody, since in the same field we could recognise a faint staining due to basal luciferase expression in almost all of the stably transfected SC. Interestingly we found strong luciferase upregulation only in SC that are in contact with axons, as judged by DAPI stained nuclear shape, which is cigar-shaped (Eldridge *et al.*, 1987). A proportion of the SC with strong upregulation have already formed myelin, as indicated by the positive MBP staining in these fibers (Fig. 2.14b). Based on the immunofluorescence data it is possible to say that the major contribution to luciferase activity is coming only from a very reduced number of SC, some of which have formed myelin.



**Figure 2.14a**

Double immunohistochemical analysis on 9.0MBP SC-neuron co-cultures. The culture was treated for 15 days with ascorbic acid. Cells were fixed and stained for MBP (red), luciferase (green) and DAPI to visualize Schwann cell nuclei.



**Figure 2.14b**

Double immunohistochemical analysis on 9.0MBP SC-neuron co-cultures. The culture was treated for 8 days with ascorbic acid. Cells were fixed and stained for luciferase (A), DAPI (B) and MBP (C). (D) shows an overlapping of the other images. Note the perfect match between the only internode evidenced by MBP staining and the SC upregulating luciferase. In panel (B) is easily recognisable a SC that upregulates luciferase but it is not expressing MBP.

In order to estimate peak enhancer activation (d12-d15) we counted, by DAPI staining, the number of SC that are in contact with neurites. We also measured how many of these SC are myelinated, by counting the number of internodes revealed by the MBP staining. First, we noted that more than 80% of SC are in contact with axons. We assumed that in this system, the total measured luciferase value, recovered with the biochemical assay, derives from two populations of SC. The first population is estimated by the percentage of SC in contact with axons but with no myelin (87.5%). Their activity is the basal one measured at d0. The other population is estimated by the percentage of SC in contact with axons and with myelin (12.5%). Their activity is unknown but significantly higher based on immunofluorescence staining (Fig. 2.14a). The weighted calculation to estimate the dilution of luciferase activity by low expressing SC is as follows:

$$\text{luc value} = (\% \text{ cells w/o myelin}) (\text{luc activity d0}) + (\% \text{ cells with myelin}) (X \text{ activity})$$

e.g.  $1107 = (0.875) (349.5) + (0.125) (X)$  or  $X=6410$  or about 20-fold induced.

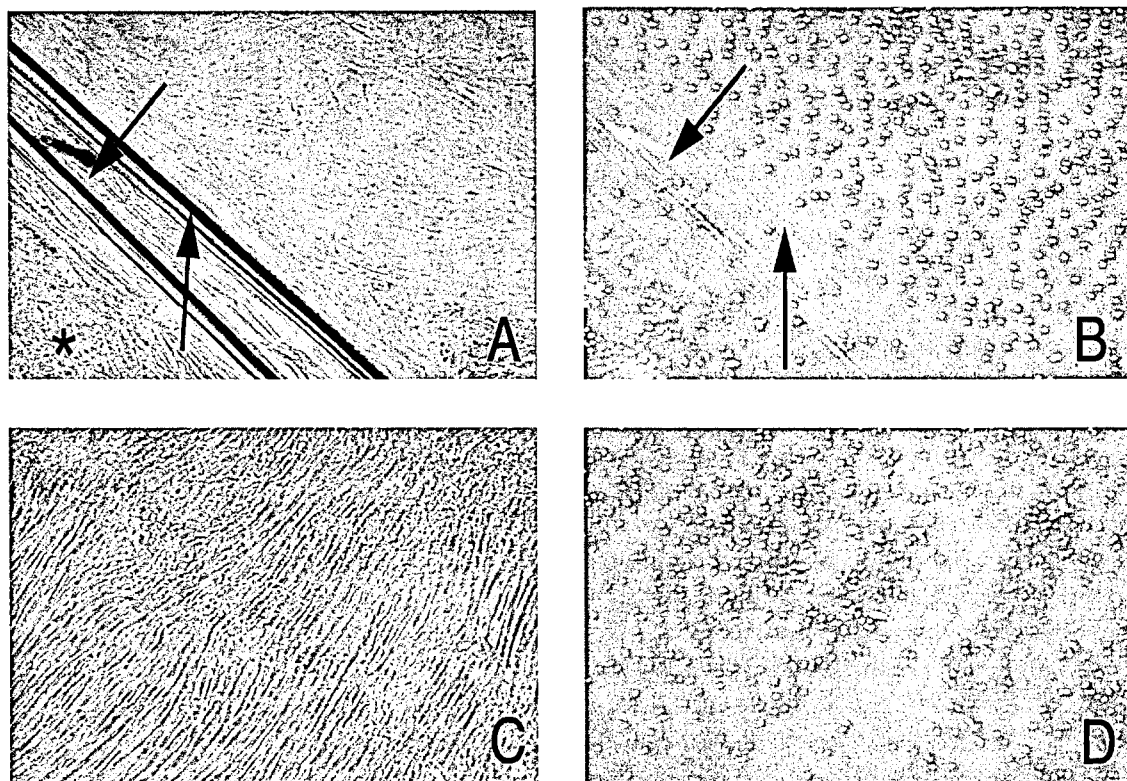
Based on this approximate calculation, we found that cells with luciferase upregulation increase the activity approximately 20-fold relative to d0. Because initially, in stable transfectants, the 9.0Kb construct has an activity 6-fold increased in respect to the 8.4Kb construct, we can deduce that the real activation of the 9.0Kb construct in the co-culture experiments, is approximately  $6 \times 20 = 120$  or about two orders of magnitude relative to constructs without SCE without axons.

The approximation we made to estimate the increase in luciferase activity in this system, is under-estimating the real induction mediated by SCE. In fact we measured the activation as the ratio between the luciferase value and protein concentration. One major factor that can influence this calculation is the change in protein concentration due to neurons and SC. We estimated the contribution given by SC to luciferase specific activity by counting SC nuclei and the change in protein concentration during all the process of myelin induction. We also controlled for the contribution in protein concentration given by neurons. We maintained, in the same set of experiments, DRG neurons in the absence of SC and we checked protein concentration at d0 and at d15. We found that while the

contribution of neurons remains almost the same between d0 and d15 (40% of total proteins), SC increased both in number and protein concentration (data not shown). Thus the specific activity we measured could be diluted.

To examine whether axons are necessary to maintain the induction of SCE activity, we cut projecting axons near their cell bodies, using a sterile scalpel (Fig. 2.15). We performed the cut 6 days after ascorbic acid treatment, when the process of myelination has already begun and luciferase is already induced, and we analysed luciferase activity 8 days after cut (Table 2), when we usually observe the maximal luciferase activity. We found that luciferase activity fell from the level of d6 to the level of d0, before myelination induction. This result strongly suggest that both induction and maintenance of SCE are axonally-dependent.

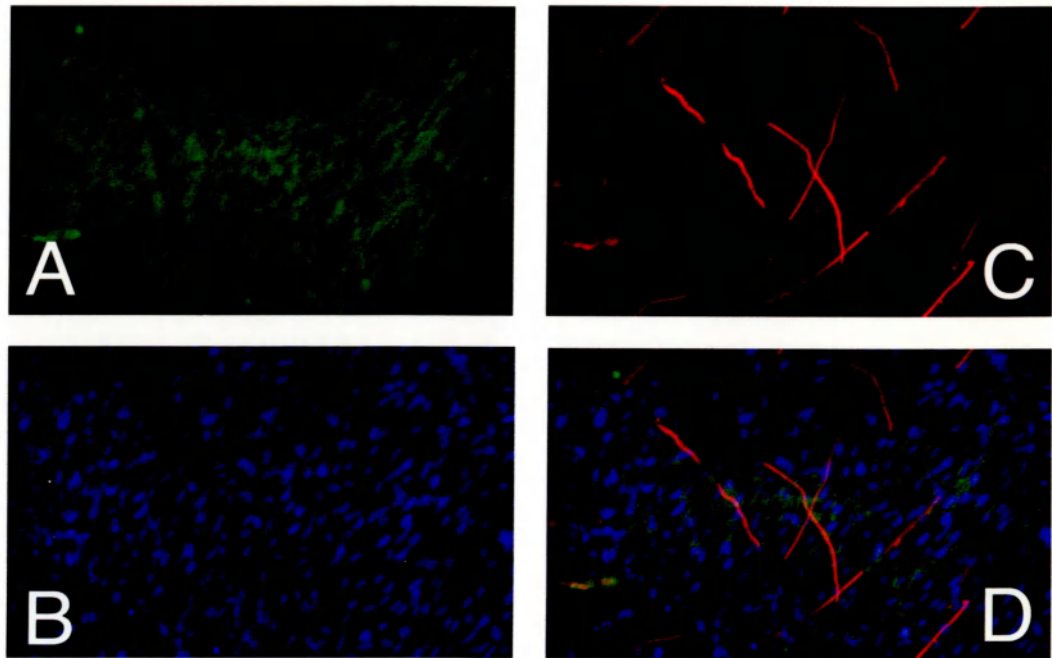




**Figure 2.15**

Contrast phase images (A) and (C) of neurons SC9.0MBP co-cultures. (B) and (D) shows the same fields stained with DAPI to visualise nuclei of SC and neurons (\*). In (A) and (B) cells were cut by scalpel, near the neuronal cell bodies as indicated by black arrows. Note the complete loss of neurites. (C) and (D) show control cultures in which neurites were not cut.

To determine where the sequence targets of axonal signals reside, we performed co-cultures experiments also with SC stably transfected with the 8.4Kb construct. It is in fact possible that not only SCE is the target for axonal signals, but also the proximal MBP promoter which is well known to be very important in directing MBP transcription (Wrabetz *et al.* 1998). We measured luciferase activity and we found that the 8.4Kb SC in the co-culture system never activated luciferase after addition of ascorbic acid (Table 2). The fact that the 8.4Kb construct is never upregulated strongly suggests that SCE contains DNA sequences that are responsive to inductive signal coming from the axons. This observation was qualitatively confirmed also by immunofluorescence (Fig. 2.16). In fact luciferase staining on the 8.4MBP cultures do not reveal any SC with a bright staining.



**Figure 2.16**

Double immunohistochemical analysis on 8.4MBP SC-neuron co-cultures. The culture was treated for 14 days with ascorbic acid. Cells were fixed and stained for, luciferase (A), DAPI (B) and MBP (C): In (D) the three images were overlapped. Note that there is no bright luciferase staining in any SC (A), whereas many fibers had already myelinated.

From these culture experiments we can conclude that axons are fundamental to recapitulate the activation observed in transgenic mice. Moreover we found that SCE is a target of axonal signals that induce MBP expression. This activation, acting on these 588nt, is in fact present only in the 9.0Kb stably transfected SC and not in SC 8.4MBP as revealed by biochemical assays and in vitro cut experiments.

Our analyses, both in transient and stable tranfectants, and even more with the co-culture system, describe a valid model in which it is possible to investigate the mechanism of MBP transcriptional regulation, as well as the axonal signals responsible for this regulation.

## Chapter 3

### DISCUSSION

#### *MBP TRANSCRIPTION IN VIVO*

Many groups have investigated the transcriptional regulation of myelin genes and in particular of the MBP gene. The approaches used in these analyses involved both classical in vitro studies as well as in vivo analyses of transgenic mice. Unfortunately due to the difficulty of setting up an in vitro system that can recapitulate the complex and precise mechanisms of MBP regulation, not information has come from the analyses of transgenic mice (Forghani *et al.*, 2001; Foran and Peterson, 1992; Goujet-Zalc *et al.*, 1993; Goverman *et al.*, 1993; Gow *et al.*, 1992; Hayes *et al.*, 1992; Jensen *et al.*, 1993; Katsuki *et al.*, 1988; Kimura *et al.*, 1989; Miskimins *et al.*, 1992; Readhead *et al.*, 1987; Zingernakel *et al.*, 1990). One of the major advantages of performing these studies in transgenic mice is that the regulation of the transgene is influenced by the same environmental factors that normally control the expression of the endogenous gene. In particular we showed in transgenic mice that axons are fundamental in regulating MBP transcription (Wrabetz *et al.*, 1998). The same studies performed in transgenic mice also showed that SC and OL use different transcription mechanisms.

#### *ADVANTAGES OF AN IN VITRO SYSTEM TO STUDY MBP REGULATION*

Analyses in transgenic mice primarily allow the identification of cis-acting sequences and not of trans-acting factors. Moreover these studies can not be easily manipulated in order to obtain a dissection of transcription mechanisms. To completely understand the mechanisms of transcriptional regulation of a gene, more simplified systems are required, like cell culture systems. The choice of the correct cell system in which to analyse the expression of a gene is particularly relevant in the case of MBP. In

fact previous in vitro studies were performed in cells that do not transcribe MBP and produced inconsistent results (reviewed in Wrabetz *et al.*, 1993 and in Taveggia *et al.*, 1998).

To identify the elements that can account for the differential MBP regulation in the CNS and PNS, we took advantage of the in vivo analyses done in transgenic mice and we tested, both by functional and biochemical analyses, the same constructs previously used to generate transgenic mice. We showed not only that in primary OL and SC culture do they recapitulate the pattern of expression observed in transgenic mice, but we also identified binding sites that could account for the differential regulation in CNS and in PNS.

However our system does not fully recapitulate the expression of the MBP gene, in particular in SC. In fact in our transient transfection studies we observed an activation between 3.1Kb and the start site of transcription that is not present in transgenic mice (Forghani *et al.*, 2001). It is possible that between 6.0Kb and 3.1Kb of the MBP promoter there is a silencer whose activity is recognised only in much more physiological conditions, such as transgenic mice. In transgenic mice DNA sequences are in fact integrated into the genome and moreover they are influenced by the same axonal signals that normally control myelin gene expression. The fact that in stably transfected SC, the 3.1Kb construct maintains a strong activity even if it is reduced, when compared to transient transfection analysis, strongly argue that chromatin is not sufficient and axons are important not only to modulate SCE amplitude (see below), but they also actively regulate MBP promoter (compare Fig. 2.3b and Fig. 2.11).

### *SCE CHARACTERISTICS*

Our transient transfection studies showed that 9.0Kb of the MBP promoter are specifically active only in primary cultures of OL and SC, confirming the cell-specific activation of this promoter in our cell model. Moreover we found two regions relevant for luciferase expression: a distal sequence, between 9.0Kb and 8.4Kb both in SC and in OL, and the proximal MBP promoter in OL. The importance of the proximal MBP promoter in directing the expression of the gene is well known also from previous studies (Wrabetz *et al.*, 1993; Taveggia *et al.*, 1998; Haas *et al.*, 1993; Haas *et al.*, 1995). In our functional studies we also showed that the region between 9.0Kb and

8.4Kb is an enhancer only in SC, further confirming that the mechanisms of transcriptional regulation differ between SC and OL (see below).

In order to identify both cis-acting sequences and trans-acting factors responsible for this differential activation, we followed two parallel approaches. We used both biochemical and functional analysis of transversion mutations and random internal deletions. The results obtained with these two approaches are not concordant, strongly suggesting that there is a combinatorial effect mediated by many transcription factors that can bind at different regions of SCE.

Since SCE behaves differently in SC and OL we made the assumption that specific differential binding could explain this. In fact, the biochemical analyses showed that there is a different set of interactions in SCE by SC as compared to OL nuclear extracts. In particular, we found 4 regions that are protected only in SC nuclear extracts, but not in brain or liver. However we could not rule out that other protected regions are important in regulating SCE. Particularly interesting are those regions protected both in SC and in brain. It is in fact possible that SC and OL contain the same set of transcription factors, but their different activity is mediated by the presence of a different subset of co-factors, in SC and OL, that differentially regulates the transcription of the MBP gene in these two glial cell types.

To explore the cellular origin of the differential bindings in SC as compared to OL, it would be useful to perform DNase I footprinting analyses using nuclear extracts prepared from OL and not from whole brain. In fact brains contain a mixture of cells and OL represent only a small part of them. One possibility would be to use nuclear extracts prepared from CG4 cells. These cells constitute a population of OL that can be differentiated in vitro by the addition of conditioned medium prepared from B104 neuroblastoma cell line (Louis *et al.*, 1992). However DNase I footprinting experiments require a large amount of nuclear extract and the quantity we obtained from differentiated CG4 was insufficient. Due to these technical limitations, we used CG4 nuclear extracts in EMSA analyses to verify the differential bindings, observed between SC and brain and we found that they are different in 3 out of 4 regions previously identified.

Specific mutations of these sites in the functional analyses were not able to assign a cell-specific role to any of these regions. In fact we found some effect in

different regions and, moreover, those regions had a more pronounced effect in OL and not in SC as expected (see Fig. 2.9a and Fig. 2.9b). From these functional experiments we can conclude that multiple regions are necessary for SCE activity. This is not surprising in fact; many enhancers require combinatorial interactions among distinct regulatory elements. The activity of the intact enhancer is distinct from that of individual elements. This was shown for the virus inducible enhancer of the human interferon  $\beta$  gene, in which none of the overlapping regulatory elements found can function on its own, but only a combinatorial effect of them can give a complete functionality (Du *et al.*, 1993).

The discrepancy between these functional studies and our biochemical analyses is emphasized also by transversion of PPAR binding site which has no effect in either primary SC or OL. From previous studies we expected that not only PPAR, but also Krox-20, whose consensus binding site is present in region 4, would play a role in OL and SC respectively (Grannemann *et al.*, 1998; Topilko *et al.*, 1994). Our results argue against a possible role for PPAR on SCE in inducing MBP transcription. It is still possible that the low amplitude we observe is not sufficient to assign an effective role to this mutation. Also *in vivo*, in transgenic mice, A. Peterson and colleagues (2001) mutated the consensus-binding site for the Krox-20 transcription factor, but this did not abolish the activity of the reporter gene (see below).

Since binding by nuclear extracts did not predict regions that mediate cell-specific effects, we also tried to dissect SCE by random internal deletions. But even in this case we were not able to assign to a specific region inside SCE a fundamental role in directing the expression of MBP. In fact the results we obtained were not in agreement with those of the biochemical assays. The maximum reduction we observed lies in a region that does not contain any protected element identified by previous biochemical assays. Moreover this effect is stronger in primary OL and not in SC. All these results strongly suggest that the enhancer activity of SCE represents a combinatorial mechanism mediated by several transcription factors.

One of the major problems we had in our cell culture system, was the defect in the overall amplitude. In general the activation observed in our *in vitro* system is not as high as the one observed in transgenic mice and this could obscure our ability to recognise cell-specific activation by sub-regions. An explanation for this difference



could lie in the fact that SC we currently used in our cell culture have the characteristics of pre-myelinating SC, and the addition of forskolin, an analogue of cAMP that is thought to partially mimic axonal signals (Lemke and Chao, 1988), can upregulate the expression of MBP only 2-3 fold above background. This strongly implies that some elements, that are fundamental to enhance transcription of not only MBP but also other myelin genes, may not be activated.

#### *REGULATION IN TRANS OF SCE*

To overcome this lack in amplitude we studied the effect of two external cues that are present in transgenic mice but not in transiently transfected plasmids in SC: the chromatin context and more importantly, the absence of axons. The analyses of our primary SC stable transfectants revealed that even for MBP, chromatin plays an important role on SCE induction. In fact, when integrated in the genome, SCE increases activation up to 6-fold. A comparison of the activation of deletion constructs, 8.4Kb and 3.1Kb, relative to the promoterless pXP1 control, in stable versus transient transfection, shows that this 6-fold activation depends on SCE, not effective repression of cis-acting elements proximal to SCE. The importance of chromatin in controlling transcription of genes is underlined by many studies. It is in fact becoming clear that chromatin is a dynamic and active structure in regulating transcription (Lemon and Tjian, 2000). Distinct mechanisms are involved in modulating chromatin: some of them function as motors to disrupt nucleosomes or as enzymatic machinery to chemically modify histones thereby facilitating promoter accessibility (Kwok *et al.*, 1994; Yang W-M *et al.*, 1996; Utley *et al.*, 1998).

#### *AXONAL REGULATION OF SCE AS DETERMINED IN MYELINATING CO-CULTURES*

More interestingly we found that axons are fundamental for induction of the expression of the MBP promoter. In fact when co-cultured with DRG neurites, our stably transfected SC can increase the activity 2 order of magnitudes above that induced by the presence of forskolin. Axons are well known to be necessary to produce robust transcription of myelin genes, including MBP, in both the CNS and PNS. MBP expression is regulated by axons during development and after axonal injury (Kidd *et*

*al.*, 1990; McPhylemy *et al.*, 1990; Scherer *et al.*, 1992). This is particularly relevant in the case of the proximal MBP promoter (Wrabetz *et al.*, 1998). In the CNS, OPC prepared from transgenic mice bearing 256nt of the proximal MBP promoter fused to *lacZ* and cultured in vitro do not express  $\beta$ -galactosidase while they can normally differentiate. On the contrary, if co-cultured with neurons, they can activate the *lacZ* expression (Lubetzki *et al.*, 1993).

The DRG neuron-SC co-culture is a well established system in which myelination can be induced by the addition of ascorbic acid in the presence of serum (Wood *et al.*, 1990). This system has many advantages if compared to studies performed in vivo in transgenic mice. In fact a precise analysis of how myelin genes are induced during myelination in the early phases of post-natal nerve development is particularly difficult because SC are not synchronized. Thus, some SC are still sorting axons and achieving proper relationships with axons, while others have already produced compact myelin. In contrast, in these co-cultures, we can induce myelination relatively synchronously and therefore precisely monitor how luciferase activation occurs both before and during myelination. Moreover, by using this method we were also able to discriminate between the necessity of simple axonal contact or the requirement of induction of myelination to achieve luciferase activation.

A second advantage of co-cultures using permanently transfected SC with luciferase constructs is axotomy. Until now in fact all the studies performed to understand the influence of axons on myelin genes were performed in vivo, in mice. However the stability of the *lacZ* reporter gene normally used in transgenic mice is too elevated in order to precisely correlate the decay after axonal injury with the decay in the activity of promoters. On the contrary luciferase is characterised by a rapid degradation of the protein, in fact it has a very rapid half-life that in mammalian cells is approximately of 3 hours (Thompson *et al.*, 1991), allowing us to analyse the effect on its activity after axotomy.

Thus, we were able to perform in vitro axotomy on 9.0Kb co-cultures, as in transgenic mice, showing that luciferase induction in this system is strictly controlled by the presence of the axon and confirming that 9.0Kb of the MBP promoter are axonally modulated. Axonal regulation of this region of the promoter is particularly important because many transcription factors involved in the myelination process were shown to

be axonally regulated. In particular Krox-20, after in vivo transection in adult mice, is downregulated strongly suggesting that it is positively regulated by signals coming from the axons (Topilko *et al.*, 1994). Even Oct-6 nerve crush and transection experiments showed that it is axonally regulated (Scherer *et al.* 1994b). To confirm in vivo the axonal regulation of SCE it will be interesting to perform crush and cut experiments on transgenic mice bearing the hsp68 promoter driven by SCE. Preliminary cut experiments performed in adult transgenic mice containing the 9.0Kb construct showed that *lacZ* is downregulated, but it is not reactivated during re-myelination (A. Peterson, unpublished result). This loss of reactivation can be due to a mosaicism effect of the transgene. Further experiments need to be done in order to investigate whether only SCE and not the proximal MBP promoter is axonally regulated also in vivo, as suggested by our in vitro results.

Finally, we found that SCE is the target of luciferase activation. In fact stably transfected SC containing the 8.4Kb construct do not activate the reporter gene in this co-culture system as revealed by both the biochemical assay and immunofluorescence analyses. This is not the only enhancer specific for SC and dependent on axons. Recently Mandemakers and colleagues localised a SC enhancer 12Kb downstream of the Oct-6 transcription initiation site (Mandemakers *et al.*, 2000). This enhancer, which is axonally regulated, can drive Oct-6 expression that rescues the peripheral nerve phenotype observed in Oct-6 null mice, and specifically directs the heterologous hsp68 promoter in peripheral nerve. To see whether these two DNA sequences have common cis-acting elements, we compared their sequences, but we were not able to find any similarities at the level of sequence.

Re-evaluation of the cis-acting sequences identified by footprinting and EMSA analyses is particularly interesting, because we expect that some are axonally controlled and the co-culture system constitutes a good model in which evaluate their function. We can not exclude that some of the transcription factors identified in this region, but previously discarded by transient transfection analysis, could be important to mediate SCE activity. In particular the signal scan analysis on the MBP enhancer revealed a consensus-binding site for Krox-20 in region 4. We confirmed by supershift analysis the binding of Krox-20 in this region (data not shown). The role played by this transcription factor was explored in vivo in transgenic mice. Peterson and colleagues

showed that it is neither necessary nor sufficient for qualitative activation (Forghani *et al.*, 2001). However they never performed a quantitative analyses of *lacZ* activity on these mice, and we can not exclude the possibility that this transcription factor can partially induce SCE. To more fully explore the role of Krox-20, it will be interesting to perform a quantitative analyses on these transgenic mice, as we did for the proximal MBP promoter (Wrabetz *et al.*, 1998) or to analyse the effect of mutation in this specific site on luciferase expression in our co-culture system; in both cases axonal signals activate SCE.

### *SCE ACTIVATION MOST CLOSELY PARALLELS MYELINOGENESIS*

In our analyses we found that to obtain an upregulation in luciferase activity we have to induce myelination. Simple contact with axons before addition of ascorbic acid was insufficient to increase luciferase activity. Another confirmation of the correlation between luciferase expression and myelination process came from the parallel increase in luciferase protein, detected with the biochemical assay, and the increasing in number of MBP fibers positive by immunofluorescence analyses. These results parallel previous analyses performed in this culture system on myelin gene expression. The first myelin segments observed in this system by immunofluorescence and positive for MAG, appear 3-4 days after ascorbic acid addition (Owens and Bunge 1989), but after 8 days myelin sheaths are considerably longer and complete, and they stain positive for MBP used as a marker of compact myelin (Zanazzi *et al.*, 2001). MAG is the first myelin protein accumulated also in vivo, while MBP is revealed only after the myelin sheath contains several turns (Hahn *et al.*, 1987).

### *QUANTIFICATION OF SCE INDUCTION BY AXONS*

From our analyses we found that the biological stimulus to obtain substantial luciferase activation is the induction of myelination. However even if the number of fibers strongly positive for luciferase parallel those positive for MBP as detected by immunofluorescence, the biochemical analyses revealed that the absolute values measured after d15 of ascorbic acid treatment are only 2-3 fold induced above that of stable integration in chromatin.

The most likely explanation for this discrepancy is suggested by immunofluorescence analysis for luciferase. The majority of cells contact axons, but do not upregulate luciferase, nor form myelin. A minority of cells do upregulate luciferase and form myelin. This is sensible, because up-regulation of MBP transcription (reported by luciferase) just precedes myelin formation. Our weighting calculation aimed to adjust for the diluting effect of the majority (70%) of uninduced cells (20% of cells do not contact axons) and it shows that the robustly induced cells (10%) activate luciferase expression 150-fold over the basal level in constructs without SCE and without axons.

This is a rough approximation, and may be an underestimate for several reasons. First, when comparing the specific activity of luciferase from 9.0Kb with axons to the 8.4Kb without axons, the proteins are assumed to be similarly from SC, but in co-cultures, neurons contribute 40% of protein, reducing the luciferase specific activity proportionately. Second, the weighting calculation assumes that the proportion of SC in the two categories is stable after d12-d15. But we counted SC directly and we found that they increase 2.5-fold between d0 and d15. We measured proteins in parallel and they increase proportionately. Since most of these cells are dividing, they are not myelinating, and will tend to dilute luciferase activity, while contributing protein. Thus the luciferase peak at d12-d15 is an underestimate.

Other explanations for reduced induction in the population of SC in co-culture include a specific effect of plasmid integration sites on myelin gene expression. But this is much less likely; in fact our stably transfected SC can myelinate and the myelin sheaths are normally compacted, as shown both by electron microscopy and immunofluorescence. Moreover we did not find a significant difference in the number of fibers that are myelinated between our stably SC and wild type SC used as a control (data not shown).

It should not be surprising that a majority of cells are non-induced, in fact it is possible that not all SC are competent for immediate luciferase induction. The process of myelination, even in this culture system, requires that SC exit the cell cycle, take a 1:1 relationship with neurites, allow basal lamina formation and induce the generation of myelin. The signals involved in this process are not completely understood, but this is not an all or none mechanism. This is underlined by the fact that even in mature

cultures the majority of nerve fibers are ensheathed but not myelinated (Salzer and Bunge, 1980).

To indirectly control for these changes and to monitor changes in protein concentration that can affect the calculation of luciferase specific activity, we estimated the SC number in our co-culture and we found that they still increase even during myelination induction. This is in partial agreement with previous studies performed on SC proliferation in this system (Salzer and Bunge, 1980). Even if the system used in this study was a little different, the authors described an initial burst in SC proliferation, due to the presence of neurons, and a subsequent decrease in its rate. Differences include that we made nuclei counting as well as protein determination on cultures performed on 35mm collagen-coated plastic dishes, while all previous studies that checked for SC proliferation rate in this system, were performed in collagen coated coverslips. The amount of SC added back to re-populate the culture is different and it is possible that the rate of proliferation is influenced by the initial numbers of plated SC. In addition they found that even after myelination, proliferation continues at lower rate in the peripheral region of the culture. It is not surprising that we found an increase in SC number, in fact the difference can be due to the geometry of our culture system.

We also showed that the activation is present only after the myelination process is induced, strongly supporting the idea that this is the correct system in which it is possible to investigate mechanisms of transcriptional regulation of myelin genes.

#### *DIFFERENTIAL TRANSCRIPTIONAL REGULATION IN SC AS COMPARED TO OL*

These studies, beside the identification of promoter sequences, confirmed that although MBP is expressed in both central and peripheral myelin, the mechanisms that govern its transcription are completely different in OL as compared to SC. Supporting the idea of the existence of different mechanisms came from *in vivo* analyses of the entire 9.0Kb of the MBP promoter (Forghani *et al.*, 2001). Another confirmation that the mechanisms of transcriptional regulation of the MBP gene are different between SC and OL came also from the analysis of Nkx2.2 transcription factor null mutants, in which the MBP expression is affected only in OL but not in DRG satellite cells (which usually behave like SC) (Qi *et al.*, 2001). Even the different developmental origins of SC and OL support the idea that even if they have similar function and they are

expressing a similar set of myelin proteins, they could have a different subset of transcription factors that are responsible for their specific function. The analysis of the MBP promoter is particularly interesting, because it is present in both CNS and PNS but it is clearly differentially regulated at the transcriptional level.

Interestingly, the only region in which we found the same binding in SC as well as OL, is region 3 in which there is a consensus-binding site for a member of the PPAR family of transcription factors. These are ligand-activated transcription factors composed of at least three isoforms ( $\alpha, \beta/\delta, \gamma$ ) belonging to the family of orphan receptors (Kliwer *et al.*, 1999). They are involved in the regulation of lipid metabolism, production and homeostasis. The  $\beta/\delta$  isoform is expressed in immature OL (Granneman *et al.*, 1998) and treatment of cultured OPC with specific PPAR $\delta$  agonists induces an enrichment in membrane formation (Saluja *et al.*, 2001). The fact that we found a common binding site for this family of transcription factor in SC as well as in OL is not arguing against a possible role of PPAR in SC. The mechanism of MBP activation, as for other genes, is composed by many transcription factors and it is possible that PPAR is a common one present both in SC and in OL, but the specificity of SCE behaviour is given by unidentified co-factors in SC as compared to OL.

Nevertheless it is also possible that PPAR plays a different role in SC and in OL. Many transcription factors in fact mediate different functionality, like activation or repression, depending on different conditions such as cellular amount, the presence of various domains as well as interactions with other transcription factors. Examples of these transcription factors are WT1, p53, Pax-5. WT1, the Wilms tumor suppressor protein, possesses independent activation and repression domains that may interact with different cellular proteins (Rauscher *et al.*, 1990). On the contrary in p53 different functions are mediated by different cellular concentrations, in fact it can activate at high concentrations and repress at lower levels (Kristjuhan *et al.*, 1995). The same mechanism has been described for another transcription factor Pax-5, which at low level activates transcription from high affinity binding sites but at higher concentrations represses transcription from low affinity binding sites (Wallin *et al.*, 1998).

The existence of these different mechanisms is particularly relevant not only for myelination, but also for re-myelination. In fact SC re-myelinate better than OL after nerve damage and the mechanisms that account for these differences in myelination are

likely to be the same also in re-myelination. During re-myelination, SC dedifferentiate to a pro-myelinating stage and they turn on the same transcriptional factors that are involved in their development. (Ghislain *et al.*, 2002). Comparing the transcriptional mechanisms that govern the behaviour of this enhancer can be indirectly important to clarify the mechanisms that control re-myelination events. However our analysis strongly suggests that only with co-cultures is it possible to correctly estimate the mechanisms involved in MBP transcription, which are relevant not only in myelination but also in re-myelination mechanisms at least in PNS.

### CONCLUSIONS AND FUTURE DIRECTIONS

In our studies we described a system in which it is possible to analyse MBP regulation in detail. We were in fact able to functionally dissect 9.0Kb of the MBP promoter. More interestingly we showed that a distal region located at the 5'-end of the promoter is a specific SC enhancer (SCE), whose function is mediated by a series of combinatorial components. We also showed that MBP transcriptional mechanisms differ between SC and OL not only at the level of the proximal promoter, but also in SCE. The setting up of a co-culture system between our stably transfected SC and DRG neurites, allowed us to recapitulate the full activation observed in vivo in transgenic mice. We also showed that not only 9.0Kb of the MBP promoter are axonally regulated, but that SCE is a target sequence for a signal coming from the axons that are responsible for a strong induction of the MBP gene during myelination.

Our ability to completely restore the activation observed in transgenic mice with the co-culture system can be used to further investigate SCE. We can in fact create stably transfected SC bearing both random SCE deletions and transversion mutations, and use these SC to re-populate DRG neurites in the co-culture model. Since the co-culture model is a more physiological system, and since we can exactly control the myelination process, we can use this system to further dissect SCE and identify regions inside it responsible for the activation of the MBP gene.

Our analyses also showed that luciferase induction occurs only in those SC that are strictly in contact with axons. Based on this observation we can hypothesise that the axonal signal/s responsible for reporter gene induction is present on the axonal membrane. In principle, we can prepare axonal membranes and add them to our 9.0Kb



stably transfected SC. If our hypothesis is correct we should be able to restore the same activation observed in our co-culture experiments. We can also consider the possibility to fractionate this membrane preparation in order to characterise the molecule/s that are responsible for MBP induction.

## Chapter 4

# MATERIALS AND METHODS

### 4.1 PLASMID CONSTRUCTS

All plasmids containing the firefly luciferase gene were generated by cloning the elements in the promoterless pXP1 construct, derived from a pSV vector, containing the  $\beta$ -lactamase gene, an *E. coli* origin of replication and a sequence derived from SV40 with multiple cloning sites (Nordeen SK, 1988). All MBP promoter fragments were derived from a pBluescript SK<sup>-</sup> plasmid containing 9.0Kb of the mouse MBP promoter (a generous gift from A. Peterson).

Restriction and modification enzymes were provided by Promega Corp., or New England Biolabs Inc. and used according to manufacturer's instructions.

- *pX9.0MBP* plasmid was generated by removing 9.0Kb of the MBP promoter from the original plasmid using SacII/SalI restriction enzymes. The SacII extremity was filled-in using T4 DNA polymerase and the fragment, after gel purification, was cloned into the pXP1 vector previously cut with SmaI/SalI restriction enzymes.
- *pX8.4KbMBP* plasmid was generated from the *pX9.0MBP* plasmid. The plasmid was cut using NaeI restriction enzyme. The NaeI fragment (511bp) was removed, gel purified and the plasmid was re-ligated.
- *pX6.0MBP* plasmid was generated from the *pX9.0MBP* plasmid. It was cut using KpnI restriction enzyme. The KpnI fragment, containing the region from 9.0Kb to 6.0Kb of the MBP promoter and part of the polylinker of pXP1, was gel extracted and the plasmid re-ligated.

- *pX3.1MBP* was generated by cutting the original plasmid from Peterson using EcoRV/XbaI restriction enzymes. The XbaI extremity was filled-in using the DNA Polymerase I Large (Klenow) fragment enzyme. The fragment was gel purified and cloned into the pXP1 vector cut at the SmaI restriction site and dephosphorylated.
- *pX1.9MBP* plasmid was generated from the pX3.1MBP plasmid. A 1.9Kb fragment was created by cutting it at SacI/SalI sites and, after gel purification, it was cloned into the pXP1 vector cut at SacI/SalI restriction enzymes.
- *pX256MBP* was generated from pX9.0MBP. The 256nt fragment was created by cutting the original plasmid with StuI/HindIII and, after gel purification, it was cloned into the recipient vector pXP1 previously cut at SmaI/HindIII sites.
- *pXP0* plasmid, containing 1.1Kb of the P0 promoter, was generated by cutting the P0CAT3 plasmid using HindIII restriction enzyme. The fragment was gel purified and cloned into the recipient vector pXP1 cut at HindIII site and dephosphorylated.
- *pXP0E* and *pXP0Er* were generated by cloning SCE, in both orientations, upstream of pXP0 plasmid. SCE fragment was prepared from the original Peterson plasmid by cutting it with SacI restriction enzyme. The fragment was gel purified and cloned into the SacI site upstream of the 1.1Kb region of the P0 promoter. Restriction enzyme digestions determined the correct orientations.
- *pT109EMBP* and *pT109ErMBP* were created by cloning SCE, in both orientations, in the SacI site upstream of pT109luc. This plasmid contains 109bp of the promoter of the Thymidine Kinase gene upstream of transcription start site of pXP1 plasmid. Restriction enzyme digestions determined the correct orientations.
- *pX256EMBP* and *pX256ErMBP* were created by cloning the SacI fragment containing SCE, in both orientations, into the SacI site upstream of pX256MBP plasmid. Restriction enzyme digestions determined the correct orientations.

- *pLOXluc* was created to generate a probe for the Southern blot analyses on genomic DNA prepared from stably transfected SC. The luciferase fragment XbaI/BsrGI (+128 to +571) was cut, gel purified and cloned in the XbaI/BsrGI site of the pLOX(BsrGI) plasmid available in laboratory.

### *SCE INTERNAL DELETIONS*

We created a series of internal deletions of approximately 100bp inside SCE (see Fig2.10a). All deletions were initially produced in pBluescript SK(+) containing SCE, and then cloned in the original context of pX9.0MBP. Four out of six deletions were generated using unique restriction sites in SCE, while two of them ( $\Delta d$  and  $\Delta e$ ) were generated by PCR and then checked by sequence analysis.

In particular:

$\Delta a$ : comprises the deletion from +1(SacII) to +103bp(BstEII)

$\Delta b$ : comprises the deletion from +103(BstEII) to +204bp(StuI)

$\Delta c$ : comprises the deletion from +204(StuI) to +290bp(BglII)

$\Delta d$ : comprises the deletion from +290(BglII) to +400bp

$\Delta e$ : comprises the deletion from +400 to +519bp(NaeI)

$\Delta f$ : comprises the deletion from +519(NaeI) to +588bp(SacI)

- $\Delta a$ : the deletion was created by cutting the pBluescript containing SCE with SacII and BstEII restriction enzymes. Both ends were filled-in by T4 DNA polymerase and, after gel purification, ligated.
- $\Delta b$ : the deletion was created by cutting the pBluescript containing SCE, with BstEII/StuI restriction enzymes. The BstEII site was filled-in by DNA Polymerase I Large (Klenow) fragment. The plasmid was gel purified and then ligated.
- $\Delta c$ : the deletion was created by cutting the pBluescript containing SCE, with BglII/StuI restriction enzymes. The BglII extremity was blunted by DNA Polymerase I Large (Klenow) fragment. The plasmid was gel purified and then ligated.

- $\Delta d$ : the deletion was generated by PCR using as template pBluescript SK (+) plasmid containing SCE cloned in the antisense orientation. Primers used in the reaction were designed in order to have a deletion from 290nt to 400nt (see PCR section). Deleted SCE was confirmed by sequence analysis.
- $\Delta e$ : the deletion was generated by PCR using as template a pBluescript SK (+) plasmid containing SCE cloned in the antisense orientation. Primers used in the reaction were designed in order to have a deletion from 400nt to 519nt (see PCR section). Deletion was confirmed by sequence analysis.
- $\Delta f$ : the deletion was generated in three steps. We first subcloned the region from -9.1Kb to -7.2Kb of the MBP promoter in pXP1 promoterless plasmid containing SCE cloned in the antisense orientation. In the second step we cut this plasmid using *Ecl136II*/*NaeI* restriction enzymes and after gel purification, it was ligated. In this second plasmid we created the final deletion. In the third step we recovered the deletion and, by cutting the recipient plasmid *Ecl136II*/*XhoI*, we cloned it in pX9.0MBP previously cut with *XhoI*/*BamHI* and filled-in.

Deletions from  $\Delta a$  to  $\Delta e$  were removed from pBluescript recipients using *BamHI*/*MluI* and cloned in the pX9.0MBP previously cut with the same restriction enzymes.

### *SCE MUTATIONS*

Mutations were generated by PCR in a pBluescript SK(+) plasmid and sequenced (see PCR section). SCE was divided in two parts and subcloned in pBluescript SK(+) plasmid. After mutagenesis, each fragment, containing the mutated site, was removed from pBluescript SK(+) using *BamHI*/*MluI* restriction enzymes and cloned in the pX9.0MBP previously cut with the same restriction enzymes.

### *PROXIMAL AND DISTAL DNASE I HYPERSENSITIVE PROBE*

The proximal probe was generated by PCR using primers based on the sequence of the mouse Golli MBP exon 5C (Campagnoni *et al.*, 1993). After amplification, the

DNA was inserted in a pBluescript SK(+) plasmid. The distal probe contains 0.2Kb of the MBP promoter located at -12.6Kb of the MBP mouse promoter. This region was obtained from a plasmid kindly provided by A. Peterson. The fragment was prepared by digestion with BamHI/PstI restriction enzymes, gel purified and cloned in the pBluescript SK(+) plasmid previously cut with BamHI/PstI.

All plasmids were prepared for transfection experiments, with a Quiagen Maxi kit plasmid (Quiagen, Italy) according to manufacturer's instructions. Usually X11 Blue host bacteria were grown overnight in 500ml of LB medium, and the day after plasmid DNA was extracted. DNA preparation and concentration were determined both by restriction enzyme digestion, and spectrophotometer measurement at a wavelength of 260nm (Ultrospec 2000, Pharmacia Biotech).

LB medium (1L):

- 10g Bacto Tryptone
- 5g Yeast Extract
- 10g NaCl

The pH was adjusted to 7.0 with NaOH, and the medium was sterilised by autoclaving.

## 4.2 POLYMERASE CHAIN REACTION (PCR)

### *DNase I HYPERSENSITIVE PROBE*

Primers used in the amplification of mouse genomic DNA to generate the proximal probe for the DNA hypersensitive assay are:

sense primer: 5'-TTGGATCCTCCACGGCTCTAG-3'

antisense primer: 5'-GGAAGCTTTGTTAGCAAGTGAGA-3'

Reaction is produced in a final volume of 25µl, and it contains:

- 2.5ul of 10x PCR buffer 10mM MgCl<sub>2</sub>
- 1µl of dNTP 10mM
- 1µl of primer 1 50pmol/µl

- 1µl of primer 2 50pmol/µl
- 0.25µl of Taq DNA polymerase 5µ/µl (Applied Biosystems)
- 1µl of mouse genomic DNA

PCR conditions are:

denaturation: 94°C x 30"

renaturation: 55°C x 1'

polymerisation: 72°C x 1'

All steps were repeated for 25 cycles, and at the end the reaction was left 10' at 72°C to complete the polymerisation step. At the end templates were cut at HindIII/BamHI sites and cloned in pBluescript SK(+) previously cut with the same restriction enzymes.

### *MUTAGENESIS*

Mutagenesis was generated by nucleotide transversions using PCR techniques. We first amplify the template with an external primer designed to it, and an internal one, which contains the mutation. In the second round of PCR the template produced in the first PCR was amplified using only external primers, in order to generate the mutation. All mutations were confirmed by sequence analysis.

Internal primers used in the amplification are:

- region 1: (nt 33 - nt 42)

sense: 5'-CTTTCATCTCGTACTCCTTTCTGTCATAGCAAGTCCAC-3'

antisense: 5'-ACAGAAAGGAGTACGAGATGAAAGGCATGTGGCTGCCG-3'

- region 2: (nt 112 - nt 127)

sense: 5'-GGTGACCCACCTAAACTTAGTAACGCGGCAGAAGTATTCCATCA-3'

antisense: 5'-TCTGCCGCGTTACTAAGTTTAGGTGGGTCACCTGGGAGGGCACA-3'

- region 3: (nt 372 - nt 380)

sense: 5'-CTTAACAAGTCAAGTGCCCCAGACCTCCTCCTTCA-3'

antisense: 5'-TGGGGCACTTGACTTGTTAAGTGGCACCCAGCCAT-3'

The external primers used are:

sense: 5'-ACCCTCACTAAAGGGAACAAAAGC-3'

antisense primer: 5'-GAGGTCGACGGTATCGATAAGCTT-3'

Both reactions were produced in a final volume of 100µl, and the reaction mixture contains:

- 10µl of 10x PCR buffer 15mM MgCl<sub>2</sub>
- 2µl of dNTP 10mM
- 1µl of primer 1 200pmol/µl
- 1µl of primer 2 200pmol/µl
- 0.5µl of Taq DNA polymerase 5µ/µl (Applied Biosystems)
- 1µl of DNA template 1µg/µl

Conditions of the cycle used in the first PCR are:

denaturation: 94°C x 1'

renaturation: 50°C x 2'

polymerisation: 72°C x 3'

All steps were repeated for 25 cycles, and at the end the reaction was left 10' at 72°C to complete the polymerisation step.

Conditions of the cycle used in the second PCR are:

denaturation: 94°C x 1'

renaturation: 50 °C x 2'

polymerisation: 72°C x 3'

All steps were repeated for 25 cycles, and at the end the reaction was left 10' at 72°C to complete the polymerisation step.

### *DELETIONS*

Deletions inside SCE are generated with two different PCRs on a pBluescript SK (+) plasmid containing SCE. In the first PCR we amplified the template using an external primer designed to it, and an internal one in which half of the primer can exactly



match the template, whereas the other half is complementary to the region downstream of the one we deleted. In the second round of PCR the template produced in the first was amplified using only external primers, in order to generate the deletion. All deletions were confirmed by sequence analysis.

Internal primers are:

$\Delta d$ :

sense primer: 5'-CCAGGTAGGAGCCAGGTTCTGGTGACAGTATCCGGG-3'

antisense primer: 5'-CCCGGATACTGTCACCAGAACCTGGCTCCACCTGG-3'

$\Delta e$ :

sense primer: 5'-CCCAGACCTCCTCCTTCAGGCACCCTCGCACCCACC-3'

antisense primer: 5'-GGTGGGTGCGAGGGTGCCTGAAGGAGGAGGTCTGGG-3'

External primers are:

sense: 5'-ACCCTCACTAAAGGGAACAAAAGC-3'

antisense primer: 5'-GAGGTGCGACGGTATCGATAAGCTT-3'

Both reactions are produced in a final volume of 100 $\mu$ l, and the reaction mixture contains:

- 10 $\mu$ l of 10x PCR buffer 15mM MgCl<sub>2</sub>
- 2 $\mu$ l of dNTP 10mM
- 1 $\mu$ l of primer 1 200pmol/ $\mu$ l
- 1 $\mu$ l of primer 2 200pmol/ $\mu$ l
- 0.5 $\mu$ l of Taq DNA polymerase 5U/ $\mu$ l (Applied Biosystems)
- 1 $\mu$ l of DNA template 1 $\mu$ g/ $\mu$ l

Conditions of the cycle used in the first PCR are:

denaturation: 94°C x 1'

renaturation: 55°C x 2'

polymerisation: 72°C x 3'

All steps were repeated for 25 cycles, and at the end the reaction was left 10' at 72°C to complete the polymerisation step.

Conditions of the cycle used in the second PCR are:

denaturation: 94°C x 1'

renaturation: 58°C x 2'

polymerisation: 72°C x 3'

All steps were repeated 25 cycles, and at the end the reaction was left 10' at 72°C to complete the polymerisation step.

#### **4.3 NUCLEAR EXTRACT PREPARATION**

##### *TISSUES*

Nuclei used in the EMSA analysis and in the DNaseI footprinting assay were dissected from 30 day rats, whereas nuclei used in DNase I hypersensitivity assay were obtained from 30 day CD1 mice. After dissection tissues were washed with PBS 1X to remove all remaining blood, resuspended in homogenisation buffer (1ml/1g of tissue) and minced with scissors. Tissues were put in a potter homogeniser containing 12.5ml of homogenisation buffer + 2.5ml of tissue and they were homogenised with 2-3 strokes at 800rpm. Nuclear integrity was checked with trypan blue staining. The homogenate is centrifuged for 1 hour at 4°C at 24000rpm using an SW28 Beckman rotor in a Beckman ultracentrifuge. The pellet containing nuclei was recovered and washed twice in 5ml of swelling buffer plus protease inhibitors and 1/10 of PAL-P. Nuclei were centrifuged 10' at 4°C at 2000rpm. At this point nuclei used in the DNase I hypersensitivity assay, were resuspended in W solution plus 50% glycerol and stored at -20°C to perform the assay; whereas nuclei derived from rats were processed to obtain nuclear extracts. To prepare nuclear extracts, they were resuspended in 2-3 vol of extraction buffer plus protease inhibitors and left on ice 30'. After centrifugation 30' at 14000rpm at 4°C the supernatant was recovered and dialysed against dialysis buffer for 5 hours at 4°C. At the end it was aliquoted and stored in liquid nitrogen. Protein concentration was checked with the Biorad Assay according to manufacturer's instructions.

## *CELLS*

Approximately 400 million Schwann cells were grown to confluence in standard growing conditions and left in the presence of 20 $\mu$ M forskolin three days before collecting them. Cells were trypsinized and washed three times in PBS 1x to remove all remaining media and then processed to obtain nuclei. Cells were passed through a dounce (pestle A) homogeniser approximately 80-times to obtain free nuclei, whose integrity was checked with trypan blue. Nuclei were then processed, as described before, to prepare nuclear extracts.

Homogenisation buffer:	- 2.0M Sucrose
	- 10mM Hepes pH7.6
	- 15mM KCl
	- 1mM EDTA
	- 0.15mM Spermine
	- 0.5mM Spermidine
	- 1:100 v:v TPL 100x mix
	- 0.5mM DTT
	- 0.5mM PMSF

TPL 100x mix:	- 2.1mg/ml Aprotinin
	- 70 $\mu$ g/ml Pepstatin
	- 70 $\mu$ g/ml Leupeptin
	- 10mM Hepes pH7.8

Swelling buffer:	- 20mM Hepes pH7.8
	- 1mM MgCl <sub>2</sub>
	- 0.5mM CaCl <sub>2</sub>
	- 1mM DTT
	- 1mM PMSF

PAL-P:	- 40mM Tris pH8.0
	- 1mM MgCl <sub>2</sub>

Extraction buffer:

- 100mM Tris pH 7.9
- 420mM NaCl
- 5% Glycerol
- 5mM MgCl<sub>2</sub>
- 1mM EDTA
- 1mM DTT
- 0.5mM PMSF

Dialysis buffer:

- 20mM Hepes pH7.8
- 20% Glycerol
- 100mM KCl
- 0.2mM EDTA
- 1mM DTT
- 0.5mM PMSF

#### **4.4 DNase I HYPERSENSITIVITY**

Nuclei were washed in RSB buffer three times to remove all remaining glycerol, and then processed with DNase I. Usually  $2 \times 10^7$  nuclei were digested with 0.05 to 20U of DNase I (Boehringer-Mannheim) 10' at 37°C in a total volume of 2 ml. Reactions were stopped by the addition of 1x DRO-Stop solution and left 1 hour at 37°C, after the addition of RNase I at a concentration of 100µg/ml. DNA was extracted with Proteinase K leaving the samples at 50°C overnight. Samples were then processed three times with phenol/chloroform, precipitated and resuspended in 100µl of TEpH8.0. The extent of digestion was determined by electrophoresis of an aliquot of DNA on a 1% (w/v) agarose gel in 1x TBE buffer.

RSB buffer:

- 10mM Tris pH7.5
- 10mM NaCl
- 3mM MgCl<sub>2</sub>
- 300mM Sucrose

Solution W:	- 60mMKCl
	- 15mM NaCl
	- 12.5mM Tris pH7
	- 2.5mM Tris pH8
	- 320mM Sucrose
	- 0.15mM Spermine
	- 0.5mM Spermidine
	- 0.05mg/ml DTT
5x Dro-Stop solution:	- 40mM EDTA pH8.0
	- 0.5% Lauril Sarcosyne
	- 200µg/ml Proteinase K

#### **4.5 SOUTHERN ANALYSIS**

Genomic DNA was completely cut using the appropriate restriction enzymes, and run on a 1% (w/v) agarose gel in 1x TAE buffer. For Southern blot analysis in DNase I hypersensitivity assays, DNA was digested with BamHI restriction enzyme; whereas to characterise pools of stably transfected SC, DNA was digested with XbaI restriction enzyme. The gel was denatured 45' in 0.5M NaOH/1.5M NaCl, and renatured 45' in 1.0M Tris pH8.0/1.5M NaCl. DNA was transferred overnight onto a cellulose membrane (Duralon, Stratagene) through capillarity in SSC 20x. The day after DNA was fixed on the membrane by UV-cross linking (Stratagene). The filter was incubated with probe overnight at 42°C, in 50% Formamide (Fluka), 5x SSPE, 1%SDS, 5x Denhardt's, 100µg/ml ssDNA. The day after the filter was washed three times by increasing the stringency to remove non-specific signals. The last wash was performed at 65°C in 0.2xSSC, 0.1%SDS. The membrane was exposed to Kodak XAR film overnight at -80°C, with an intensifying screen.

#### 4.6 DNase I FOOTPRINTING

Fragments used in the DNaseI footprinting experiments were generated by subdividing SCE in 5 overlapping regions using unique restriction enzyme sites. All the fragments were blunted and cloned into the SmaI site of pBluescript SK+. The orientation was then determined by sequencing. In particular:

- fragment 1: from NcoI site (+72bp) to BglII site (+290bp)
- fragment 2: from BglII site (+290bp) to MluI site (+570bp)
- fragment 3: from SacII site (+1bp) to BseRI site (+195bp)
- fragment 4: from BseRI site (+195bp) to BseRI site (+390bp)
- fragment 5: from BseRI site (+390bp) to SacI site (+588bp)

40-80µg of nuclear extract were incubated with the Klenow end-labelled probe and digested with 500ng of DNaseI (Boehringer Mannheim, dissolved in LSBD buffer). The binding reaction was carried out in a final volume of 50µl containing 1/10 vol. of KM buffer, 2µg of poly-[d(I-C)], nuclear extract (eventually diluted in LSDB buffer) and the probe (30,000cpm). All reactions were maintained for 45' on ice. DNase I digestion was performed at room temperature by adding the DNase for 2'. The reaction was then immediately stopped with 100µl of Stop solution and extracted with 1:1 (vol/vol) phenol:chloroform. DNA was precipitated with the addition of 1µg of tRNA as a carrier. All reactions were resolved on a 6% polyacrylamide denaturing gel. The gel is dried and exposed to Kodak XAR film overnight at -80°C, with an intensifying screen. As a ladder we used a G+A reaction of the same fragment treated using the Maxam-Gilbert method.

#### *G+A LADDER PREPARATION*

Approximately 200,000cpm of the same probe are put in a final vol. of 10µl of dH<sub>2</sub>O plus 1µg/µl of ssDNA (Sigma) as carrier. 25µl of pure Formic Acid (Fluka) was added to DNA 5' at room temperature, to get chemical modifications. The reaction was stopped with 200µl of HZ solution. The chemically modified probe is precipitated twice and nucleotides were modified with 1M Piperidine (Fluka) 30' at 90°C.

Piperidine is removed by lyophilisation in a speedvac overnight, and washed many times to remove it completely the day after.

LSDB buffer:

- 50mM KCl
- 1mM MgCl<sub>2</sub>
- 25% Glycerol
- 20mM Tris pH8.0
- 1mM DTT

KM buffer:

- 300mM KCl
- 20mM MgCl<sub>2</sub>

HZ solution:

- 30mM EDTA pH 8.0
- 200mM NaCl
- 1% SDS
- 100ug/ml yeast tRNA

HZ Stop solution:

- 300mM Sodium acetate pH7.5
- 0.1mM EDTA pH 8.0
- 25ug/ml yeast tRNA

#### **4.7 EMSA AND PPAR SUPERSHIFT**

Synthetic double strand oligonucleotides containing potential binding sites were commercially produced. Complementary pairs of oligonucleotides were annealed in 150mM NaCl in TE by heating 2' at 85°C, followed by slow cooling to room temperature. Oligonucleotides were end-labelled by filling or replacing 5' protruding ends with DNA Klenow polymerase enzyme. Unincorporated radiolabelled oligonucleotides were removed by column purification. The binding reaction was carried out in a final volume of 20µl containing 100mM or 50mM KCl, 2µg of poly-[d(I-C)], nuclear extracts (eventually diluted in dialysis buffer) and the probe (60,000cpm). All

reactions were maintained 45' on ice with a pre-incubation of 30' when a cold competitor was added. Binding was resolved on a 2xTAE, 8% or 5% acrylamide non-denaturing gel. The dried gel is exposed to Kodak XAR film overnight at -80°C, with an intensifying screen. EMSA supershift is made in the same way as standard EMSA except that the binding reaction, and antibody incubation, were performed 20' at room temperature. The antibody is a commercially available antiserum directed against mouse PPAR (Alexis Biochemical Corp).

Oligonucleotides used in EMSA analysis are:

- region 1 (nt 24-nt 55):

sense: 5'-GCCTTTCATAGATGCAGAATTTCTGTCATAGC-3'

antisense: 5'-GCTATGACAGAAATTCTGCATCTATGAAAGGC-3'

- - region 2 (nt 103-nt 143):

sense: 5'-GGTGACCCCAAGCCCAGGCTGCCAGCGGCAGAAAGTATTCC-3'

antisense: 5'-GGAATACTTCTGCCGCTGGCAGCCTGGGCTTGGGGTCACC-3'

- - region 3 (nt 362-nt 396):

sense: 5'-GCCACTTAACCCTGACCTGGCCCCAGACCTCCTCC-3'

antisense: 5'-GGAGGAGGTCTGGGGCCAGGTCAGGGTTAAGTGGC-3'

- - region 4 (nt 530-nt 569):

sense: 5'-CGCACCCACCCACGCTTAATGGATATGGAGACATTGAGCC-3'

antisense: 5'-GGCTCAATGTCTCCATATCCATTAAGCGTGGGTGGGTGCG-3'

As unrelated competitor we used oligonucleotides encompassing region (nt-149-nt-116) of human MBP proximal promoter.

sense: 5'-GCGCACAGCGGACCCGAAGAATGCCTGGCAGGAT-3'

antisense: 5'-ATCCTGCCAGGCATTCTTCGGGTCCGCTGTGCGC-3'

As a positive control in EMSA supershift we used synthetic oligonucleotides designed from the acyl-CoA oxidase promoter previously shown to contain a PPAR responsive element (Schulman *et al.*, 1998):

sense: 5'-GACAGGGGACCAGGACAAAGGTCACGTTCTGGGAGT-3'

antisense: 5'-ACTCCCGAACGTGACCTTAGTCCTGGTCCCCTGTC-3'



## 4.8 CELL CULTURE AND TRANSIENT TRANSFECTION

### *SCHWANN CELLS*

Schwann cells were prepared from sciatic nerve of P3 Sprague-Dawley rats (Charles River). Sciatic nerves were minced and incubated at 37°C, 30' in 0.1% (wt/vol) Collagenase type IV (Boehringer Mannheim, Germany) and in 0.2% trypsin (GIBCO) prepared in Hank's balance salt solution. Reactions were stopped by the addition of 1/10 of heat inactivated Foetal Calf Serum (FCS). After mild triturating with a Pasteur pipette, cells were cultured in 100-mm plastic dishes covered by poly-L-Lysine (Sigma). Schwann cells were plated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS. Contaminating fibroblasts were eliminated by treatments with the antimetabolic agent cytosine arabinoside ( $10^{-5}$  M, Sigma) and antibody-complement-mediated cell lysis, using the monoclonal anti-Thy 1.1 antibody (undiluted hybridoma culture supernatant, American Tissue Culture Collection), and rabbit complement (Sigma). Cells are then passaged three times to expand in DMEM + 10% FCS + 2 $\mu$ M Forskolin (Calbiochem) and crude GGF prepared from bovine pituitaries (Brookes *et al.*, 1980; Porter *et al.*, 1986). The day before transfection  $2.5 \times 10^5$  cells were plated in a 60mm plastic dish covered by poly-L-Lysine (Sigma).

### *OLIGODENDROCYTES*

Oligodendrocyte precursors were prepared from brains of P6 Sprague-Dawley rats (Charles River). After mechanical dissociation, brains were incubated 30' at 37°C in 100 $\mu$ g/ml of DNase I (Sigma) and 0.1% trypsin (Sigma) prepared in PBS 1x. The reaction is inhibited by the addition of Calf Serum. Cells were then filtered through filters of 140 $\mu$ m and 25 $\mu$ m in diameter. Approximately  $3 \times 10^6$  cells were cultured in 60mm plates (Nalgene) in MEM and supplemented medium. The day after medium was switched to a defined medium (F12 and supplements), and left in this medium for 4 days. Precursors were differentiated to mature oligodendrocytes by the addition of 1% human PPP (Poor Platelet Plasma) and 8% PDGF (R&D Systems).

- MEM supplement medium:
- Eagle's Minimal Essential medium (GIBCO)
  - 1x Non essential Aminoacids (GIBCO)
  - 5x Calf serum
  - 5x Foetal Calf serum
  - 1x Penicillin/Streptomycin solution (GIBCO)
- F12 supplement medium:
- F12 medium (GIBCO)
  - 2x MEM aminoacid (GIBCO)
  - 1x MEM vitamins (GIBCO)
  - 100u/ml Insulin (Sigma)
  - 10mg/ml Human Transferrin (Sigma)
  - 0.5mg/ml Hydrocortisone (Sigma)
  - 10nM T3 (Sigma)
  - 3nM Sodium Selenite (Collaborative Research)
  - 1x Penicillin/Streptomycin solution (GIBCO)

*HeLa CELLS, L CELLS, Cos7 CELLS*

All cell lines were maintained in a medium containing DMEM supplemented with 10%FCS, 2mM glutamine and 100µg/ml Penicillin/Streptomycin solution. The day before transfection,  $2.5 \times 10^5$  cells were plated on 60mm plastic dishes.

*TRANSFECTION*

All cells were transfected using  $\text{CaPO}_4$  precipitation as previously described (Graham and VanDerErb, 1973). An hour before transfection, cells were put in DMEM, 10%FCS, 2mM glutamine and 100µg/ml Penicillin/Streptomycin solution. Usually 10µg of DNA were transfected per dish (5µg of supercoiled pBluescript SK (+) DNA as a carrier, 2.5µg of supercoiled plasmid containing the luciferase gene as a reporter, and 2.5µg of a pRSVgal plasmid whose activity is a measure of transfection efficiency). The amount of supercoiled reporter plasmid transfected was usually normalised to molar ratio. Cells were left in the presence of the precipitate for 4 hours except SC, which were left 16 hours. Cells were washed twice with HBSS and re-fed in

the same medium used for culture for 72 hours, before testing luciferase and  $\beta$ -gal activity.

#### **4.9 LUCIFERASE ASSAY AND $\beta$ -GAL ASSAY**

Cells were washed twice with PBS 1x to remove medium. Cells were detached with a cell scraper and the addition of 200 $\mu$ l of lysis buffer per 60-mm plastic dish. After recovery all cellular debris was removed by centrifugation and the supernatant was tested for the luciferase and  $\beta$ -gal activity.

##### *LUCIFERASE ASSAY*

20 $\mu$ l of cell extract were added to 350 $\mu$ l of reaction buffer and tested in a Luminometer (Lumat LB 9501, Berthold). Approximately 100 $\mu$ l of d-Luciferin (Sigma) were injected into each sample. The activity was recovered after 10". Each sample was tested in duplicate.

Lysis buffer:	- 1% Triton X-100 (Fluka)
	- 25mM Gly-Gly solution pH7.8 (Sigma)
	- 15mM MgSO <sub>4</sub>
	- 4mM EGTA
	- 1mM DTT
Reaction buffer:	- 2.5mM Gly-Gly solution pH7.8
	- 2mM ATP (Sigma)
	- 10mM MgSO <sub>4</sub>
Injection mix:	- 20mM Gly-Gly pH7.8
	- 0.2mM d-Luciferin (Sigma)

##### *$\beta$ -GAL ASSAY*

100 $\mu$ l of each extract were added to 1ml of Z-buffer 1x and 200 $\mu$ l of 4mg/ml of o-Nitrophenyl- $\beta$ -D-Galactopyranoside (ONPG, Sigma). The reaction was then

incubated at 37°C until the sample become yellow. The reaction was stopped by the addition of 500µl of 1M NaCO<sub>3</sub> and the extent of substrate hydrolysis was measured in a spectrophotometer with a wavelength of 420nm (Ultrospec 2000, Pharmacia Biotech).

Buffer Z 10x:

- 600mM Na<sub>2</sub>HPO<sub>4</sub>
- 400mM NaH<sub>2</sub>PO<sub>4</sub>
- 100mM KCl
- 10mM MgCl<sub>2</sub>

### *PROTEIN ASSAY*

Protein concentration was measured using a Biorad Assay (Biorad) according to manufacturer's instructions. The substrate was diluted 1:5 in dH<sub>2</sub>O and protein concentration determined relative to a standard curve made with a known amount of BSA. Protein concentration was determined in a spectrophotometer with a wavelength of 595nm (Ultrospec 2000, Pharmacia Biotech).

### **4.10 SC STABLE TRANSFECTION**

Schwann cells were transfected using CaPO<sub>4</sub> precipitation as previously described. An hour before transfection, cells were put in a medium containing DMEM, 20%FCS, 2mM glutamine, 100µg/ml penicillin/streptomycin solution, 4µM forskolin and crude GGF prepared from bovine pituitaries. Usually 40µg, 30µg or 10µg of supercoiled DNA were transfected in 100mm plastic dishes coated with PLL in which, the day before, we plated 5x10<sup>5</sup> SC. We co-transfected pXP1 promoterless plasmid, pX3.1MBP, pX8.4MBP, pX9.0MBP with pMAMneo plasmid containing the neo gene for positive selection. Plasmids were transfected both at a 5:1 and at a 10:1 ratio. Selection of positive clones was made by adding to standard medium 400ug/ml of G418 (GIBCO). Selected clones, obtained in each plate, were then pooled together by passage and tested for luciferase activity, protein concentration and copy number insertions by Southern Blot. The final activity of each pool, was measured as a ratio between luciferase activity and copy number insertions.

#### 4.11 DRG-SC CO-CULTURES

Purified DRG neurons were dissociated from E15.5 Sprague-Dawley rats (Charles River) and placed in culture as previously reported (Kleitman *et al.*, 1998). After excision, DRG were treated with trypsin (0.25% in HBSS, GIBCO) and left at 37°C 45'. DRG were also mechanically dissociated through a sterile pasteur pipettes. The cell suspension (approximately 3-5 DRG per drop) was plated as a drop onto ammoniated rat collagen (0.2 mg/ml, Biomedical Tech. Inc, Stoughton, MA) – coated 35-mm plates (Nalgene) or 12 mm glass coverslips in C<sub>B10</sub> media and left overnight. The day after medium was changed to E<sub>2</sub>F medium and cycled on C<sub>B10</sub> every other feeding for 2 weeks to remove non-neuronal cells. 2.5 weeks after neuronal seeding, Schwann cells were added to plates and coverslips (75,000 each one). Co-cultures were kept in DM medium for 1 week to allow re-population of neurites. To initiate myelination, co-cultures were treated with MF medium.

- |                          |  |
|--------------------------|--|
| C <sub>B10</sub> medium: | <ul style="list-style-type: none"> <li>- Eagle's Minimal Essential medium (GIBCO)</li> <li>- 10% FCS</li> <li>- 5mg/ml D-glucose (Sigma)</li> <li>- 50ng/ml 2.5S NGF (Harlan, Indianapolis, IN)</li> </ul>   |
| E <sub>2</sub> F medium: | <ul style="list-style-type: none"> <li>- EMEM</li> <li>- 5mg/ml D-glucose (Sigma)</li> <li>- 50ng/ml 2.5S NGF</li> <li>- 5µg/ml Insulin (Sigma)</li> <li>- 10µg/ml rat Trasferrin (Jackson Immunoresearch)</li> <li>- 100µM Putrescine (Sigma)</li> <li>- 20nM Progesteron (Sigma)</li> <li>- 30nM Sodium Selenite (Becton Dickinson)</li> <li>- 10µM Uridine (Sigma)</li> <li>- 10µM FdU (Sigma)</li> </ul> |

- DM medium:
- 1:1 DMEM/F12
  - 50ng/ml 2.5S NGF
  - 5µg/ml Insulin (Sigma)
  - 10µg/ml rat Trasferrin (Jackson Immunoresearch)
  - 100µM Putrescine (Sigma)
  - 20nM Progesteron (Sigma)
  - 30nM Sodium Selenite (Becton Dickinson)
- MF medium:
- Eagle's Minimal Essential medium (GIBCO)
  - 15% FCS
  - 5mg/ml D-glucose (Sigma)
  - 50ng/ml 2.5S NGF (Harlan, Indianapolis, IN)
  - 50ug/ml Ascorbic acid (Sigma)

#### **4.12 IMMUNOFLUORESCENCE**

Cells were fixed 10' in paraformaldehyde and for 15' in cold methanol. Double staining was performed with mouse anti-myelin basic protein mAb (Roche) diluted 1:300, and goat polyclonal anti luciferase (Promega) diluted 1:100, applied 60' at room temperature. Positivity was revealed with FITC- or TRITC-conjugated secondary antibodies, and examined with fluorescence microscope (Olympus AX).

#### **NUCLEAR STAINING**

DRG co-cultures were incubated for 1' with DAPI diluted 1:2500 (Sigma) or 20' at 37°C in PBS1x with 1µg/ml of vital Hoechst (Sigma). After this period of time, cells were washed twice with sterile PBS1x and re-fed with their original medium. DAPI staining was examined with fluorescence microscope (Olympus AX), while a count of nuclei was made at a fluorescence inverted microscope (Zeiss).

#### **4.13 ELECTRON MICROSCOPY**

SC-DRG neuronal co-cultures were fixed for 20' in 2% buffered glutaraldehyde and post-fixed for two hours in 1% osmium tetroxide at room temperature. After alcohol dehydration (50-70-95-100%, 10' each), the samples were embedded in a mixture containing Epon hydrophobic resin and ethanol 100% 2:1, 16 hours at 37°C. Fixed co-cultures were then transferred for 48 hours at 60°C to facilitate the removal of plastic dishes from cells. After plastic removal, semi-thin sections (0.5µm) were stained with toluidine blue and examined by light microscopy (Olympus AX-70). Ultra-thin sections (60nm thick) were stained with uranyl acetate and lead citrate and examined by electron microscopy (Zeiss).

#### **4.14 IN VITRO AXOTOMY**

SC DRG co-cultures were cut with a disposable sterile scalpel 6 days after ascorbic acid addition. The cut was made proximal to neuronal cell bodies. Cultures were maintained in the same myelinating medium until d14, when they were processed for nuclear counting, luciferase assay and protein determination as for standard co-culture.

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